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Difluorophosphate as a ^{19}F NMR probe of erythrocyte membrane potential

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Abstract. Erythrocyte membrane potential can be estimated by measuring the transmembrane concentration (activity) distribution of a membrane-permeable ion. We present here the study of difluorophosphate (DFP) as a ^{19}F NMR probe of membrane potential. This bicarbonate and phosphate analogue has a pK_a of 3.7 ± 0.2 (SD, $n=4$) and therefore exists almost entirely as a monovalent anion at physiological pH. When it is incorporated into red cell suspensions, it gives two well resolved resonances that arise from the intra- and extracellular populations; the intracellular resonance is shifted ~ 130 Hz to higher frequency from that of the extracellular resonance. Hence the transmembrane distribution of DFP is readily assessed from a single ^{19}F NMR spectrum and the membrane potential can be calculated using the Nernst equation. The membrane potential was independent of, DFP concentration in the range 4 to 59 m*M*, and haematocrit of the cell suspensions of 31.0 to 61.4%. The membrane potential determined by using DFP was 0.94 ± 0.26 of that estimated from the transmembrane pH difference. The distribution ratios of intracellular/extracellular DFP were similar to those of the membrane potential probes, hypophosphite and trifluoroacetate. DFP was found to be transported across the membranes predominantly via the electrically-silent pathway mediated by capnophorin. Using magnetization transfer techniques, the membrane influx permeability-coefficient of cells suspended in physiological medium was determined to be $7.2 \pm 2.5 \times 10^{-6}$ cm s^{-1} (SD, $n=4$).

Key words: Difluorophosphate – Erythrocyte membrane potential – ^{19}F NMR

1. Introduction

Classically, the membrane potential of cells and organelles is measured with ion selective and non-selective microelectrodes (Goldman 1943; Kessler et al. 1976; Kamo et al. 1979). However these applications have been excluded from use in many mammalian cells because of the possible damage to cellular or subcellular membrane structures (Hoffman and Laris 1974). Incorporation of extrinsic electrochromic optical probe molecules, mainly fluorescent dyes, can avoid damaging cellular structure and hence allow the study of membrane preparations (Bashford and Smith 1979). These applications depend on the changes that occur in the optical properties of probe molecules, in response to variations of the association with membranes and macromolecules. This association can vary substantially between systems; as a result, the major difficulty encountered with optical probes is the tedious calibration of the probe signal as a function of the absolute magnitude of the membrane potential (Hoffman and Laris 1974). Alternatively, direct measurements of the transmembrane distributions of radio-actively labelled probes such as [^{14}C]thiocyanate can be used to monitor the changes of membrane potential (Deutsch and Kula 1978).

Being non-invasive, NMR spectroscopy offers a more convenient and reliable means of measuring membrane potential over those mentioned above. Probe molecules, incorporating ^{31}P and ^{19}F atoms have been used with ^{31}P and ^{19}F NMR to monitor the changes of membrane-potential of human erythrocytes (Kirk et al. 1988; London and Gabel 1989).

Difluorophosphate (DFP), a bicarbonate and phosphate analogue with a pK_a of 3.7 ± 0.2 (SD, $n=4$), bears a single negative charge at physiological pH. When incorporated into red cell suspensions its intra- and extracellular populations give rise to two well resolved resonances in ^{19}F and ^{31}P NMR spectra. This phenomenon enables the accurate estimation of transmembrane distributions of the probe molecule from the ^{19}F and ^{31}P NMR spectra, without the need to separate extracellular fluid from the cells. ^{19}F NMR has a detection sensitivity comparable to that of ^{1}H NMR, and ~ 30 fold more than that of ^{31}P and it does not have the dynamic range problems that arise with ^{1}H NMR. Therefore it provides a very satisfactory detection sensitivity for biological samples. The lack

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of natural abundance of ^{19}F atoms in biological systems is an additional advantage and the large chemical shift range of ^{19}F yields better spectral resolution than that of ^{31}P NMR. We present here estimations of membrane potential of human erythrocytes, using DFP and ^{19}F NMR.

2. Materials and methods

2.1. Solutions

DFP was purchased from Strem Chemicals (Newburyport, MA) as a hemihydrate; it contained trace amounts of monofluorophosphate, hexafluorophosphate and hydrofluoric acid as confirmed by ^{19}F NMR. Where indicated it was used, without further purification, to prepare isotonic solutions (~130 m*M*, ~pH 7 by titration of the solution with 10 N NaOH) usually containing 2 m*M* EDTA as a chelator of paramagnetic ions. DFP was purified from the commercial product (yellowish) by reacting it with an excess of P_2O_5. This converted F^- to DFP at low temperature (195 K) and pressure (7 Pa) (Bernstein et al. 1971). The osmolalities of the solutions were measured using a Wescor vapour pressure osmometer (Wescor Inc., Logan, UT). 4,4′-Dinitrostilbene-2,2′-disulfonic acid (DNDS) was supplied by Pfaltz and Bauer Co., Waterbury, CT. A 100 m*M* aqueous solution was prepared freshly and stored in the dark (Frohlich and Gunn 1981). Aliquots of the DNDS solution were added to cell suspensions to achieve a concentration of 1 m*M* to inhibit membrane exchange of monovalent anions (Passow 1986). D_2O was from the Australian Institute for Nuclear Science and Engineering, Lucas Heights, NSW; it was routinely incorporated into NMR samples (~20% v/v) for field/frequency locking (Gadian 1982).

2.2. Erythrocyte suspensions

Erythrocyte concentrates were supplied by the NSW Blood Transfusion Service within 2 days of donation by healthy volunteers. The cells were suspended in the standard anticoagulant, preservative solution, CP2D (Raftos et al. 1990). CO-gassed buffers were used in centrifugal washing of the cells (Potts et al. 1989). These buffers included Na_2HPO_4-NaH_2PO_4 (20 m*M*) buffered isotonic saline (~pH 7); Na_3citrate-citric acid isotonic buffer (~pH 7.2) with 10 m*M* KCl and 5 m*M* glucose; and isotonic saline (145 m*M* NaCl; 2 m*M* KCl and 20 m*M* glucose). Cell suspensions were then gassed with CO in a tonometer for ~15 min; this converted de oxyhaemoglobin to carboxyhaemoglobin thus minimizing the paramagnetic broadening effect of de oxyhaemoglobin on the spectral lines.

To produce a range of pH values in the cell suspensions, which contained 20 m*M* DFP, a standard volume of strong acid (300 m*M* HCl) or base (300 m*M* NaOH) was slowly added (~20 µl min^{-1}) to cells suspended in unbuffered isotonic saline. This slow addition avoided pH overshoot and possible cell damage. Haemolysates of packed cells were prepared by repeatedly freezing (in liquid nitrogen, 77 K) and thawing (310 K). The pH values of the extracellular fluids and haemolysates were determined (immediately after NMR experiments) with a microelectrode within a 37 °C water jacket (Raftos et al. 1990). The electrode unit was connected to an automatic titration unit (Radiometer, Copenhagen, Denmark).

Haematocrits of the cell suspensions were measured after NMR experiments by using a microhaematocrit centrifuge (Clements Pty Ltd, North Ryde, NSW, Australia). A −2.0% factor was applied to all the measured haematocrits to correct for extracellular fluid trapped between the cells (Dacie and Lewis 1975). Mean cell-volumes were calculated from cell counts, using a Sysmex Microcellcounter CC-130 (Toa Medical Electronics Co. Kobe, Japan), together with the haematocrit measurements.

Volume fractions of solvent water in cells were determined by a gravimetric method (Cook 1967). Cell water was evaporated by drying the cell suspensions at 95–100 °C for 24 h. The decreased weight of the cells was then used for calculating the cell water content. It is known that a small fraction of cytosolic volume is occupied by non-solvent water, i.e., the water fraction that is associated with haemoglobin (Gary-Bobo 1964). DFP is a hydrophilic anion with a small Van der Waals volume (36.54 cm^3 mol^{-1}) as calculated in accordance with the method of Bondi (1964); it is thus likely that these probe molecules are able to penetrate the water structure adjacent to the protein molecules. The total volume fraction of solvent water was therefore used as the volume fraction accessible to DFP in evaluating its intracellular concentration.

2.3. ^{19}F NMR Experiments

^{19}F NMR spectra of cell suspensions were acquired at 310 K on either a Varian XL/VXR-400 spectrometer (Varian Associates, Palo Alto, CA) with a 5-mm proton probe tuned to 376.3 MHz, or a Bruker AMX-400 spectrometer with 5-mm dedicated ^{19}F probe tuned to 376.47 MHz (Bruker Analytische Meßtechnik GmbH, Germany). A standard single pulse with a nutation angle of 90° was used to acquire the "fully relaxed" spectra. The longitudinal relaxation time (T_1) of DFP was measured using a composite-pulse technique (COMP-T_1; Levitt and Ernst 1983).

To estimate the transmembrane equilibrium exchange rate-constants of DFP, a DANTE pulse train (Morris and Freeman 1978; Potts et al. 1989) was used to saturate the intracellular DFP magnetization. The diminution of the extracellular resonance due to magnetization transfer, was then measured and used to calculate the exchange rate constants. The rate constants were also measured using the 'overdetermined' 1-D EXSY analysis (Bulliman et al. 1989).

All spectra were acquired without spinning the sample, in order to minimize sedimentation of the cells. Free induction decays (FID) were acquired into 16 K memory locations. An exponential multiplication factor of 2 Hz was routinely applied to the FID before Fourier transfor-

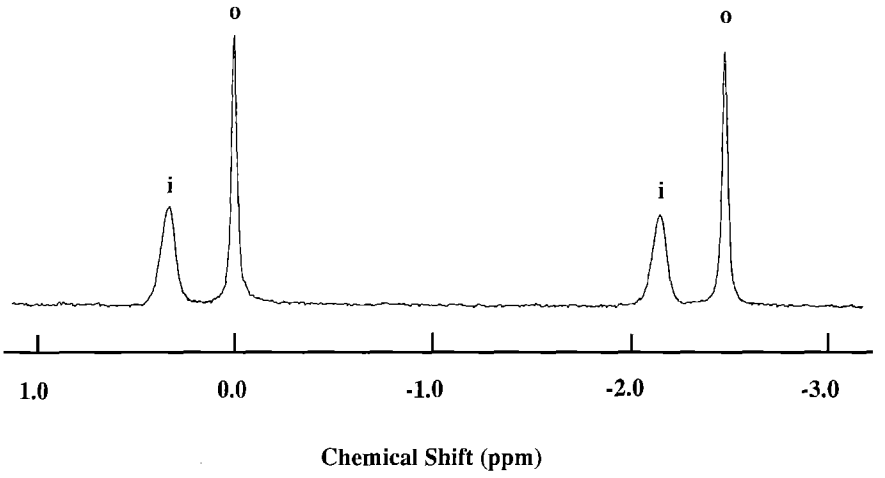

Fig. 1. "Fully relaxed" ^{19}F NMR spectrum of DFP (18 m*M*) in a red cell suspension (H_c 47%) in Na_3-citrate-citric acid (20 m*M*) buffered sucrose solution, pH 7.23. The peaks from intracellular (*i*) DFP are shifted (127 Hz) to higher frequency from those of extracellular (0) DFP. The J_{FP} of the doublet is ~960 Hz. The chemical shift of the high frequency component of the extracellular doublet was set arbitrarily to 0.000 ppm

mation. Spectral peak areas were measured by automated integration, with the integral lines covering a frequency range of ±10 times the peak-width at half-height; where the frequency difference between intra- and extracellular resonances was ~130 Hz, the integral lines were taken only to a frequency intermediate between the two peaks. This procedure enabled an estimation of >90% of the absolute area for a Lorentzian peak.

3. Results

3.1. Transmembrane distribution of DFP

A doublet (J_{FP} ~960 Hz) was evident in ^{19}F NMR spectra of DFP in aqueous solutions. When incorporated into the red cell suspensions, DFP gave two well resolved resonances, one each from the intra- and extracellular DFP; the intracellular resonance was shifted ~130 Hz to higher frequency from that of the extracellular DFP resonance (Fig. 1). Diminution of the high frequency resonance occurred with a decrease in haematocrit thus assigning it to the intracellular population; also, the high frequency resonance was very small in cell suspensions that were pretreated with 1 m*M* DNDS, an inhibitor of capnophorin-mediated transport (Passow 1986). The shift to high frequency of the intracellular resonance is thought to be mainly due to the disruption, by intracellular protein, of direct hydrogen bonding between solvent water and the fluorine atoms; these nuclei serve as "reporters" of the hydrogen-bond environment (Xu et al. 1991). The spectral line-widths of intra- and extracellular DFP in cells suspended in isotonic media were typically ~20 Hz and ~5 Hz, respectively. The logitudinal relaxation times (T_1) of intra- and extracellular DFP were measured to be 1.8±0.1 s (SD, $n=4$) and 4.5±0.3 s (SD, $n=4$), respectively. The probe concentration in the intra- and extracellular compartments of an erythrocyte suspension can be estimated from the spectral peak-areas, together with a knowledge of the haematocrit (H_c), and the cell volume fraction (V) available to the solvent (H_2O) and the solute (Kirk et al. 1988). Thus,

$$[DFP]_i/[DFP]_o = I_i(1-H_c)/(I_o V H_c), \quad (1)$$

where I_i and I_o are the spectral integrals of intra- and extracellular DFP resonances.

The pK_a of DFP was determined to be 3.7±0.2 (SD, $n=4$) by pH titration. Thus DFP exists predominantly in the monoanionic form at physiological pH. Incubation of red cells at 37 °C in phosphate buffered saline, with 1 m*M* DNDS, resulted in substantial inhibition of DFP uptake. Therefore, the predominant route of DFP transmembrane exchange is probably capnophorin. By using magnetization transfer techniques (Fig. 2), the membrane permeability-coefficient for influx into cells in the presence of physiological Cl^- (~140 m*M* extracellular) was estimated to be $7.2\pm2.5\times10^{-6}$ cm s^{-1} (SD, $n=4$). Butanol is known to fluidize cell membranes and increase the nonspecific permeability of the membrane bilayers to a number of solutes (Lieb and Stein 1986). Butan-1-ol was incorporated (concentrations ranged from 0.0 to 160 m*M*) in the cell suspensions, with haematocrits ranging from ~45% to 58%, to investigate the possibility of simple diffusion of DFP via the lipid components of the membrane. However no significant changes of DFP influx rate constants were obtained thus suggesting that simple diffusion via the membrane lipids only plays a minor role in the rapid transmembrane exchange of DFP. We concluded that DFP transport into the intracellular compartment occurs in exchange with Cl^- and HCO_3^- and establishes an electrochemical equilibrium. Therefore, the transmembrane distribution of DFP implies the membrane potential (E_{DFP}) as given by the Nernst equation (2). The equation is also written with the expression for the standard deviation, derived from the general expression for a function of several random variables (e.g., Kuchel et al. 1988):

$$E_{DFP} = -\{RT/F\}\ln\{I_i(1-H_c)/(I_o V H_c)\} \pm\{RT H_c/[F(1-H_c)]\}\{SD^2_{I_i}/I_i^2 + SD^2_{I_o}/I_o^2 + SD^2_v/V^2 + 2SD^2_{H_c}/H_c^2\}^{1/2}, \quad (2)$$

where SD with the corresponding subscript denotes the standard deviation of each individually estimated parameter; R, T and F are the standard gas constant, absolute temperature and Faraday constant, respectively.

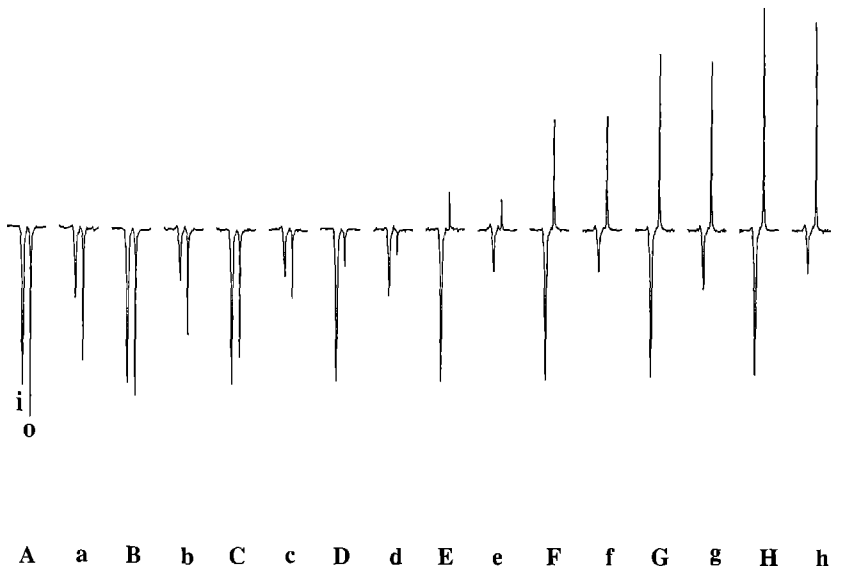

Fig. 2. ^{19}F NMR spectra of a one dimensional 'overdetermined' EXSY experiment of an erythrocyte suspension containing 20 m*M* extracellular DFP. Intracellular Cl^- was replaced by repeated exchange of Cl^- with DFP in isotonic sucrose solution, pH 7.10. Spectra were acquired at 310 K on a Bruker AMX-400 spectrometer, using an inversion transfer pulse train $[\pi/2_x - t_1 - \pi/2_x - t_m - \pi/2_{x,y,-x,-y}$ acquire $(t_2)]$. Spectra labelled with upper case letters were acquired with variable magnetization-evolution time (t_1) ranging from 0.003 s through 1/16 $\Delta\nu$, 2/16 $\Delta\nu$, 3/16 $\Delta\nu$, 4/16 $\Delta\nu$, 5/16 $\Delta\nu$, 6/16 $\Delta\nu$ and 8/16 $\Delta\nu$ s ($\Delta\nu = 83$ Hz) after the first 90° pulse, but without a "mixing time" ($t_m = 0$ s). Those labelled with lower case letters were acquired with variable magnetization evolution time as above but with $t_m = 0.8$ s. Spectra of one pair, e.g., A and a, were acquired with an identical evolution time. The integrals, peak areas, were entered into a computer program (Bulliman et al. 1989) for the evaluation of the exchange rate constants

3.2. Effect of DFP concentration and haematocrit on membrane potential

Variation of DFP concentration from 4.4 m*M* to 59.2 m*M* in red cells suspended in phosphate buffered saline with haematocrit of 33.0±0.2% (SD, $n=3$) revealed only a minor change of membrane potential (0.01 mV mM^{-1}; Table 1 A). Table 1 B shows that the increase of haematocrit of cells in isotonic saline from 31±0.5% (SD, $n=3$), to 63±0.9% (SD, $n=3$) did not give rise to any significant change of DFP membrane potential.

3.3. Anion distribution and DFP membrane potential

It has long been realized that H^+ ions passively exchange across the red cell membrane via the Jacobs-Stewart cycle (Jacobs and Stewart 1942). At electrochemical equilibrium the transmembrane pH difference is determined by the Gibbs-Donnan phenomenon (Brey 1978). Thus, measurements of transmembrane pH difference have been used to evaluate the membrane potential (Macey et al. 1978; Rottenberg 1979; Kirk et al. 1988). The electrically silent DFP exchange requires that DFP establishes a multiple-equilibrium correlation with H^+, OH^- and Cl^- ions. This correlation was examined by measuring the change of DFP distribution of cells suspended in unbuffered saline containing 1 m*M* KCl, 1 m*M* EDTA and 20 m*M* glucose with a variation of the transmembrane pH difference measured in a titration experiment. The intracellular pH was found to increase in a sigmoidal fashion (Table 2; plot of intracellular pH versus extracellular pH not shown), with an increase of extracellular pH; but the intracellular pH was consistently less than that of the extracellular solution which is consistent with previous studies (Raftos 1990). In addition, the mean cell volumes decreases from $9.9\pm0.1\times10^{-11}$ cm^3 (SD, $n=7$) to $7.4\pm0.1\times10^{-11}$ cm^3 (SD, $n=7$); this was attributed to the decrease of the volume fraction of cell water from

Table 1. Independence of DFP membrane potential on DFP concentration[a] (A) and haematocrit[b] (B)

(A)		(B)	
[DFP] (m*M*)	E_m±SD (mV)	H_c±SD %	E_m±SD (mV)
4	−0.6±1.0	31.0±0.5	−4.9±0.4
9	−1.8±1.0	36.3±0.4	−5.6±0.3
13	−4.5±1.0	47.0±0.4	−5.3±0.5
26	−1.3±1.0	47.8±0.4	−5.8±0.5
27	−1.0±1.0	51.3±0.2	−4.8±0.4
36	0.0±1.3	53.9±0.8	−4.1±0.8
44	0.1±1.0	61.4±0.2	−5.1±0.7
59	2.7±1.4		

[a] Cells were suspended in isotonic phosphate-buffered saline, pH 7.44. Haematocrits were adjusted to 33.0±0.2%
[b] Cells were suspended in isotonic saline containing 10 m*M* DFP

Table 2. Estimation of membrane potential by transmembrane pH difference and equilibrium distribution of DFP[a]

pH_i±SD	pH_0±SD	E_{pH}±SD (mV)	E_{DFP}±SD (mV)
5.98±0.03	6.28±0.01	−18.5±1.9	−21.3±1.4
6.01±0.02	6.29±0.00	−17.3±1.2	−22.3±2.5
6.15±0.01	6.44±0.01	−17.9±0.9	−24.3±2.3
6.31±0.02	6.59±0.04	−17.3±2.8	−20.8±0.2
6.36±0.01	6.70±0.03	−20.9±2.0	−22.8±2.1
6.51±0.01	6.88±0.04	−22.8±2.5	−22.3±1.5
6.62±0.01	6.98±0.02	−22.2±1.7	−28.9±1.7
6.73±0.03	7.15±0.04	−25.9±3.1	−28.1±1.2

[a] Erythrocytes from one donor were suspended in isotonic saline (1 m*M* KCl, 20 m*M* glucose and 1 m*M* EDTA) containing 20 m*M* DFP. pH_i and pH_0 denote the intra- and extracellular pH, respectively; E_{pH} and E_{DFP} denote membrane potential estimated from the transmembrane pH difference and DFP distribution, respectively

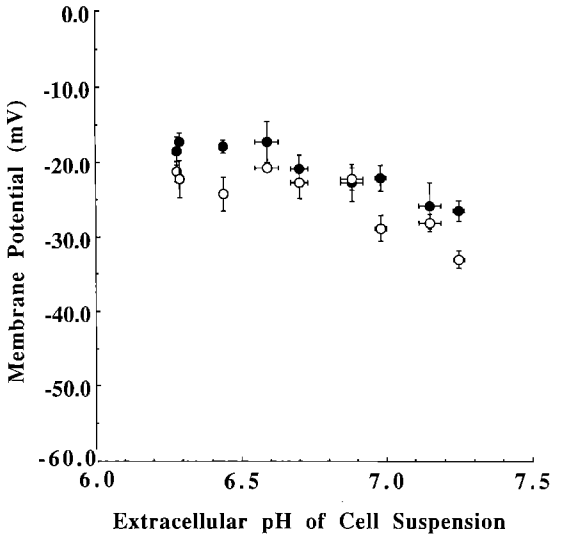

Fig. 3. pH titration of cells suspended in isotonic saline (145 m*M* NaCl, 1 m*M* KCl and 20 m*M* glucose) containing ~20 m*M* DFP. Variation of extra- and intracellular pH were achieved as described in Sect. 2.2. The membrane potential was estimated from the transmembrane pH difference (solid circles). ^{19}F NMR spectral integral values were used to evaluate the DFP membrane potential (open circles)

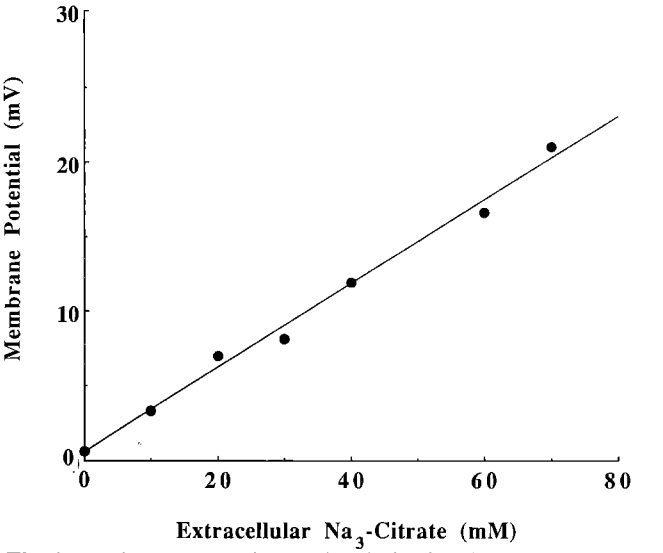

Fig. 4. Erythrocyte membrane depolarization by the membrane-impermeable ion, citrate. Cells were suspended in isotonic saline containing Na_3-citrate of various concentrations. Fully relaxed ^{19}F NMR spectra were acquired at 310 K on a Bruker AMX-400 spectrometer. The line was drawn by linear least-squares regression of the equation of a line onto the data and it yielded $E_{DFP}=0.28\,X+0.55$ ($r^2=0.991$); X denotes the extracellular citrate concentration

77.7±0.1% (SD, $n=3$) to 68.8±0.1% (SD, $n=3$). If we neglect the (supposedly minor) difference of dissociation constants and solute activity coefficients between the two compartments (Bromberg et al. 1965), the equilibrium membrane potential of the cell suspension can be estimated from the transmembrane pH difference. These differences were measured by placing a sensitive pH electrode in the extracellular solution and then in packed cells that had been ruptured by repeatedly (three times) freezing and thawing (Sect. 2.2). Figure 3 and Table 2 show the consistent changes of DFP membrane potential (E_{DFP}) along with that of the H^+ membrane potential (E_{pH}). In order to give an indication of the similarity of the estimates from the two methods linear least-squares regression of a straight line onto the graph, containing the two groups of membrane potentials, was carried out. The mean value of E_{DFP}/E_{pH} together with its standard deviation obtained from the slope of the line, of the univariate analysis, was 0.94±0.26. Therefore, within experimental error the transmembrane distribution of DFP at equilibrium appears to be governed by the Gibbs-Donnan phenomenon as also occurs with H^+ (and also OH^- and Cl^-). Therefore,

$$[DFP_i]/[DFP_o] = [OH_i^-]/[OH_o^-] = [H_o^+]/[H_i^+] = [Cl_i^-]/[Cl_o^-], \quad (3)$$

where square brackets denote molar concentration.

We compared the membrane potential estimated from the DFP distribution ratio with those estimated from hypophosphite (Kirk et al. 1988) and trifluoroacetate (London and Gabel 1989); this was carried out by incorporating these solutes into red cells suspended in hypertonic medium (osmolality 386 mOsmol kg^{-1}). The osmolality of the medium was increased to ensure sufficient resolution of the ^{31}P resonances of intra- and extracellular hypophosphite. Ratios of 2.37±0.12 (SD, $n=3$), 2.75±0.13 (SD, $n=3$) and 2.30±0.15 (SD, $n=3$) were found for the intra- to extracellular distributions of DFP, trifluoroacetate and hypophosphite ions, respectively. Thus the equilibrium membrane potentials indicated by the three probe compounds are very similar.

It is known that addition of membrane-impermeable citrate to red cell suspensions results in depolarization of the membranes (Funder and Wieth 1966); this causes a redistribution of membrane permeable anions. In the present work an increase of extracellular citrate concentration to 70 m*M* gave rise to an increase of ~21 mV in the DFP membrane potential (Fig. 4). This increase occured in a linear manner described by the equation $E_{DFP}=0.28\,X+0.55$ ($r^2=0.991$) where X represents the extracellular citrate concentration.

3.4. Effects of DFP solution on erythrocyte metabolism

The effects of DFP on erythrocyte metabolism were investigated by monitoring cellular glucose, lactate and ATP levels. Addition of an isotonic solution of DFP to a final concentration of 22 m*M* in a cell suspension (H_c ~55%) was found to reduce the rate of glucose consumption from ~0.41 mmol (l cell)$^{-1}$ h^{-1} to ~0.05 mmol (l cell)$^{-1}$ h^{-1}. The cellular ATP was depleted rapidly with a half-life of ~28 min; it remained steady in the absence of DFP. No change of lactate level was found while it accumulated at a rate of ~1.67 mmol (l cell)$^{-1}$ h^{-1} in the control study.

4. Discussion

The highly selective permeability of the erythrocyte membrane to both cations and anions is well known (Sachs et al. 1975). The extent of the membrane's selectivity for anions over cations is remarkable: the permeability to monovalent anions such as Cl^- is $\sim 10^6$ times greater than that of monovalent cations such as Na^+ and K^+ (Fostes 1977). This results from an important physiological specialization of red cells that allows the rapid exchange of CO_2, carried by blood, with the capillaries of the lungs (Jacobs and Stewart 1942; Sachs et al. 1975). Among the cation transport pathways, the one that appears to be physiologically most important is the Na^+/K^+ pump that consumes ATP (Clark 1988). Under physiological conditions, the conductive flux of such an exchange is just balanced by the fluxes of cations and anions through other pathways (Clark 1988). In contrast to the active transport of cations, anions are transported, across membranes, predominantly via the capnophorin mediated, electrically-silent, pathway (Passow 1986; Clark 1988); such a mechanism requires an obligatory 1 : 1 exchange of anions between the two cellular compartments. Physiologically, it ensures a cellular electrochemical equilibrium. However there exists a small fraction (10^{-4}) of anions that are transported via other pathways, such as pump-independent Na^+/K^+ cotransport (Clark 1988).

Membrane potential is defined as a mathematical function of the activities of ions that freely permeate the membrane from either side. For erythrocytes in physiological media, the membrane potential is determined by the permeability coefficients and the concentrations (assuming there exist no differences of activity coefficient between the two compartments) of Na^+, K^+ and Cl^-. Since the permeability coefficients of Na^+ and K^+ are $\sim 10^6$ times less than that of Cl^-, the human red cell membrane potential (E_m) is given by the Nernst potential of Cl^- (E_{Cl^-}):

$$E_m = E_{Cl^-} = (RT/F) \ln ([Cl^-]_i/[Cl^-]_o), \quad (4)$$

where all the symbols are as those defined in Sect. 2. Equation (4) thus enables the evaluation of membrane potential from the measurement of the transmembrane distribution of Cl^- (Funder and Wieth 1966). Macey et al. (1978) discussed the measurement of changes in membrane potential by determining the variation of extracellular pH while intracellular pH was unchanged. Incorporating the exogenous anions hypophosphite (Kirk et al. 1988) and trifluoroacetate (London and Gabel 1989) enables the evaluation of membrane potential from NMR measurements of the transmembrane distributions of the ions. The basis of these approaches is the rapid transport of these ions through the membranes, with the equilibrium distribution governed by the 'bulk' membrane potential.

The transmembrane distribution of DFP is readily assessed from a single ^{19}F NMR spectrum. Estimation of the DFP distribution from a "fully relaxed" NMR spectrum requires that the resonances of the two populations be well resolved; Fig. 1 shows that this is readily achieved. In Fig. 1, the separation (~ 130 Hz) of the intra- (peak width at half height $= \sim 17$ Hz) and extracellular populations (peak width at half height $= \sim 7$ Hz) ensured the unequivocal determination of the relative intra- and extracellular populations. However, attempts to estimate the distribution from a ^{31}P NMR spectrum were unsuccessful due to the incomplete resolution of the peaks from the two populations in cells of *normal volume*.

The correct estimation of membrane potential from the DFP distribution also requires that it is all NMR "visible", i.e., there exists no association to macromolecules such as haemoglobin that lead to loss of signal by fast-relaxation induced peak broadening. Measurements of the spin-spin relaxation times (T_2; results not shown) showed no evidence of DFP association with macromolecules. Studies of the red cell non-solvent-water fraction for a number of alcohols (Gary-Bobo 1964; Gary-Bobo and Solomon 1968) indicated that there exists no significant fraction of the water volume that is not accessible to a number of short chain alcohols. Similarly the total water space in the red cell appears to be accessible to DFP; this is perhaps not surprising given its small Van der Waals volume (36.54 $cm^3\ mol^{-1}$) and hydrophilic nature.

The membrane potential estimated by DFP was found to be independent of its concentration over a large range (Table 1 A). Therefore, the present method has an advantage over fluorescent dye methods which require calibration of the intensity of fluorescence with the concentration of the dye (Hoffman and Laris 1974). It was found in a separate study to be independent of haematocrit over a large range (Table 1 B). The consistency of the membrane potential estimated by the transmembrane pH difference and DFP (Table 2 and Fig. 3) verified the multi-equilibrium expressed by (3). The continued increase of intracellular pH with extracellular pH clearly demonstrated the redistribution of permeable ions (Raftos 1990; Raftos et al. 1990). This ionic redistribution results in water movement and hence cell shrinkage as the extracellular pH rises.

Addition of membrane impermeable Na-citrate causes depolarization of the membrane (Funder and Wieth 1966; Kirk et al. 1988). A ~ 21 mV increase of potential accompanied the 70 m*M* increase of extracellular Na-citrate (Fig. 4); the increase of membrane potential is similar to that found by Kirk et al. (1988). The variation of membrane potential can be explained to be the result of the decrease of extracellular Cl^-, OH^- and DFP ions; such a change of ion distribution is required to minimize the difference of transmembrane osmolality, and to maintain electrical neutrality between the inside and outside of the cells; thus the membrane potential increases.

A drawback in the use of DFP is its possible inhibition of erythrocyte energy metabolism. It was found, in the presence of 10 m*M* DFP and small amounts of contaminating F^- that glucose consumption was reduced to $\sim 5\%$ of the normal value; cellular ATP was rapidly depleted with a half-life of ~ 28 min; and accumulation of lactate, seen in the control sample, did not occur. Note that the preparations of DFP contained F^- that is known to inhibit enolase and hence the conversion of 2-phosphoglycerate to phosphoenolpyruvate of the glycolytic

pathway (Brewer 1975). Therefore the inhibition of glycolysis limits the use of DFP to cell preparations in which continuous monitoring of metabolism is not required.

In general, calculating the membrane potential of cells by measuring anion equilibrium distributions is valid only for cells in which cations have negligible permeability. The membrane potential can then be estimated by using the Nernst equation (Hladky 1977; Rottenberg 1979). It is evident when the K^+-ionophore valinomycin is incorporated into red cells (Hunziker et al. 1985) that Cl^- and thus DFP potentials are no longer equivalent to the overall membrane potential, such as may be measured with a glass micro-pipette. In other words evaluation of the membrane potential in the presence of a cation-ionophore requires the cation equilibrium concentration distribution to be taken into account (e.g., Kirk et al. 1988).

Limitations in the method also arise from the DFP traversing the membrane via capnophorin. In cells that do not have this specific and electrically-silent pathway for anion transport, diffusion of probes through the lipid bilayer of membranes becomes essential.

In comparison with trifluoroacetate (London and Gabel 1989), DFP trasverses the membrane more rapidly in establishing its transmembrane electrochemical equilibrium. For erythrocytes in physiological medium DFP gave better resolution of the ^{19}F NMR resonances ($\sim$0.35 ppm; our results) of its intra- and extracellular populations compared with those of trifluoroacetate ($\sim$0.19 ppm; London and Gabel 1989). Furthermore DFP may also be used as a ^{31}P NMR probe for membrane potential estimation. It therefore is an attractive alternative for determining membrane potential using ^{19}F and/or ^{31}P NMR spectroscopy.

In conclusion DFP was used with ^{19}F NMR to estimate the membrane potential of red cells in suspensions of high haematocrit. Many of the experimental complexities of the methods that employ fluorescent dyes or radio-isotope labelled solutes are not encountered with the present procedure. Thus, DFP may be a useful progenitor of other ^{19}F NMR probe-molecules, and the general NMR procedure may be applicable to other cell-types.

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