Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 5, pp. 305-314, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)E0019-X

Imidazol-1-ylalkanoic Acids as Extrinsic ^{1}H NMR Probes for the Determination of Intracellular pH, Extracellular pH and Cell Volume

Soledad Gil,[a] Paula Zaderenzo,[a] Fátima Cruz,[b] Sebastián Cerdán[b] and Paloma Ballesteros*[a]

[a]*Departamento de Química Orgánica y Biología, Facultad de Ciencias, UNED, 28040-Madrid, Spain*
[b]*Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029-Madrid, Spain*

Abstract—The synthesis and biological evaluation of a novel series of extrinsic probes for intracellular pH (pH_i), extracellular pH (pH_o) and cell volume determination by ^{1}H NMR is described. Imidazol-1-ylacetate, malonate, 3-glutarate and 2-succinate esters were synthesized by reaction of imidazole with α-bromo esters or with α,β-unsaturated esters. The corresponding acids were prepared by hydrolysis. Rat erythrocytes were readily permeable to methyl imidazol-1-ylacetate, moderately permeable to diethyl 2-imidazol-1-ylsuccinate and impermeable to diethyl 3-imidazol-1-yl-glutarate esters. Imidazol-1-ylacetic acid was the only acid derivative which penetrated the erythrocyte interior when added directly to the incubation medium. Transport of the permeable compounds to the erythrocyte interior was non-saturable up to 200 mM added compound. Addition of methyl imidazol-1-ylacetate or diethyl 2-imidazol-1-ylsuccinate esters to erythrocyte suspensions, resulted in hydrolysis to imidazol-1-ylacetic acid and 2-imidazol-1-ylsuccinic acid mono-ethyl ester in the intracellular and extracellular spaces, respectively. pH_i and pH_o were determined from the different chemical shifts of the H-2 proton of the acid derivatives in the intracellular ($H\text{-}2_i$) and extracellular ($H\text{-}2_o$) compartments. In addition, the relative intracellular and extracellular volumes could be calculated from the areas of the intracellular and extracellular H-2 resonances.

Introduction

Key cellular events like differentiation, response to growth factors or hormonal signal transduction have been shown to proceed with changes in intracellular pH (pH_i), extracellular pH (pH_o) and cell volume.[1-5] Progress in our understanding of the physiological roles of these three variables has been mainly derived from the development of a variety of techniques which allowed progressively more precise measurements to be made. In particular, methodologies like equilibrium distribution of radioactive weak acids and bases,[6] pH microelectrodes,[7] fluorescent probes and video microscopy[8] or NMR spectroscopy[9] were implemented.

In the last decade, the NMR approach experienced a considerable development primarily because of its ability to perform repetitive, non-invasive measurements of pH_i, pH_o and cellular volume. Among the various NMR methodologies, ^{31}P NMR spectroscopy was the most widely used,[9] taking the chemical shift of intracellular or extracellular P_i as reporter group of pH_i or pH_o, respectively, and a variety of non-ionizable phosphonates as probes of cellular volume.[10,11] Alternative ^{19}F and ^{13}C NMR techniques for the determination of pH_i were also reported[12,13] and more recently ^{19}F probes for cell volume determination have been introduced.[14] In contrast, the use of ^{1}H NMR to determine pH_i, pH_o or cellular volume received considerably less attention.[15] However, Rabenstein and Isab[16] proposed in 1982 the use of imidazole **1** as an extrinsic probe for pH_i determination by ^{1}H NMR, remaining until now as the only example of a ^{1}H NMR probe for pH_i described in the literature. The method was based on (i) the general theory of equilibrium distribution of weak acids and bases between the intracellular and extracellular environments[6]; (ii) the slow exchange (in the NMR time scale) of imidazole between the intracellular and extracellular spaces; (iii) the large titration range (0.93 ppm) of the chemical shift of the imidazole H-2 proton within the physiological pH.

In the present study, we report several modifications of the basic structure of the imidazole molecule designed to improve its performance as an extrinsic pH probe for pH_i and pH_o, and extend this approach to the simultaneous determination of the relative intra and extracellular volume in cell suspensions. We have prepared acetate, malonate, succinate or glutarate esters at position 1 of the imidazole molecule and increased the negative charge of the corresponding anions at physiological pH, by preparing the corresponding acids. In the following it will be shown that, because of more favorable pK_a's, and an increased ^{1}H NMR titration range in the imidazol H-2 resonance, some of the acid derivatives of these compounds were able to perform better than unsubstituted imidazole as extrinsic ^{1}H NMR probes. A preliminary communication has been published.[17]

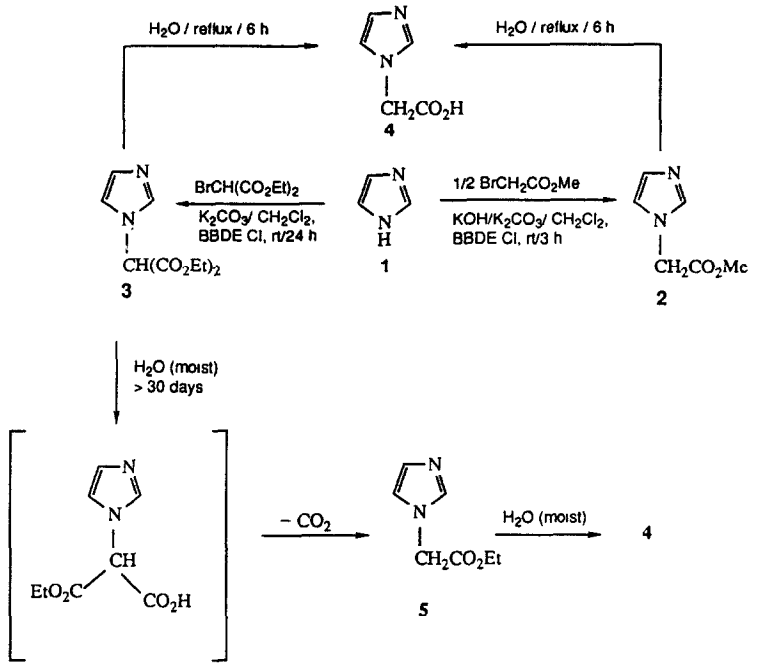
Results and Discussion

Synthesis

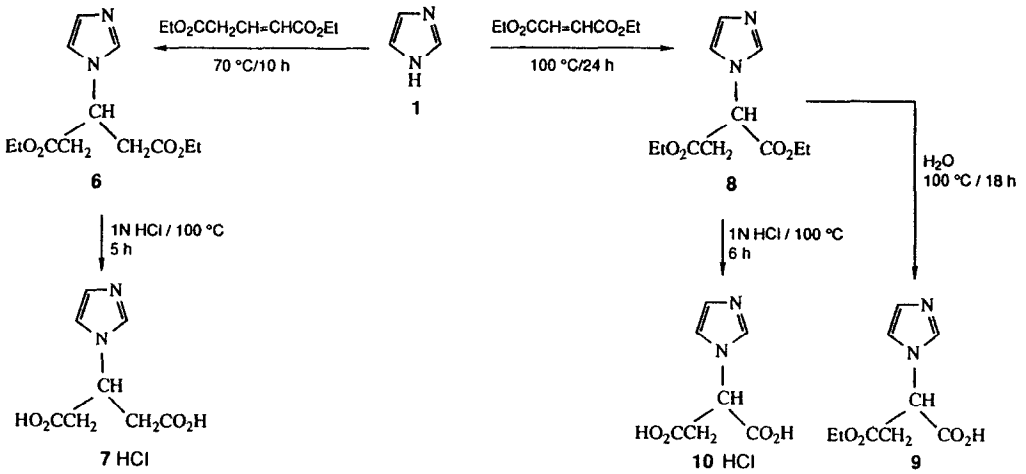
Imidazol-1-ylacetate and malonate esters (**2** and **3**) were prepared by alkylation of imidazole (**1**) either with methyl bromoacetate or diethyl bromomalonate under phase transfer catalysis (PTC) conditions, using 1,5-bis-(*N*-

benzyl-*N,N*-diethylammonium) diethylether, dichloride (BBDE Cl)[18] as new catalyst. This PTC method provided a convenient alternative to other described methods for the preparation of imidazol-1-ylacetate and malonate esters.[19] Especial precautions must be taken in the preparation of **2**, since reaction times longer than three hours result in complete hydrolysis of the ester to acid **4**. This acid was easily obtained either from **2** or **3** by hydrolysis in hot water or simply standing in a moist atmosphere. From the product mixture obtained in the partial hydrolysis of **3**, compounds **4** and ethyl imidazol-1-ylacetate (**5**) were isolated. Formation of these compounds can be explained by a combination of spontaneous hydrolysis and decarboxylation *in situ* (Scheme I). The imidazol-1-ylmalonic half-ester intermediate undergoes a rapid decarboxylation favoured by the presence of the imidazole ring.

Diethyl 3-imidazol-1-ylglutarate (**6**) could not be prepared by the PTC alkylation method because diethyl 3-bromoglutarate[20] underwent an elimination process in the basic medium to give diethyl glutaconate. However, compound **6** was easily obtained by addition of imidazole to this α,β-unsaturated ester. This type of addition reaction was used to prepare diethyl 2-imidazol-1-ylsuccinate (**8**), with diethyl fumarate or maleate as α,β-unsaturated esters. Compound **8** experienced a regioselective hydrolysis to its half-ester form **9** after heating in water. This hydrolysis, similar to that mentioned above for acetate **2** and malonate **3**, is also observed at room temperature when compound **8** was stored in a moist medium. However, hydrolysis of ester **6** to diacid **7** and complete hydrolysis of **8** to diacid **10** required stronger conditions such as diluted hydrochloric acid solution (Scheme II).



Scheme I.



Scheme II.

Compounds **2–10** were fully characterized by spectroscopic methods and elemental analysis.[17] ^{1}H NMR data of compound **5** in $CDCl_3$ were in agreement with those previously reported.[19a,b] All compounds showed high solubility in water, a very convenient property to implement biological experiments. Table 1 summarizes the most relevant ^{1}H NMR characteristics of esters **2**, **3**, **6**, and **8** and acids **4**, **7**, **9**, and **10** in D_2O, providing a frame for the discussion of the results presented in the next sections.

Apparent pK_a (pK'_a) values of the H-2 proton and lipophilic character

The performance of an extrinsic ^{1}H NMR probe for pH_i depends on its pH-titration behavior and on its permeability characteristics. Ideally, pH_i (and pH_o) determinations in biological systems are favored by compounds having (i) a pK'_a in the physiological region and (ii) a large ^{1}H NMR titration range. Additionally, the probe must be able to penetrate the cellular interior, a property related in many cases to its hydrophobicity or lipophilic character. Protonation behavior and permeability properties are related, since the ionization state determines the relative electric charge of the molecule, and charged molecules are thought not to pass readily through biological membranes.[6] Table 2 summarizes pK'_a values of the H-2 proton of compounds **1–10** as well as their lipophilic character ($\log k'_0$).

The pK'_a of the H-2 proton in compounds **1–4** and **6–10** was determined by ^{1}H NMR spectroscopy. pH titrations (22 °C) were performed using 10 mM solutions of the appropriate compound in D_2O adjusting the pH with the addition of NaOD or DCl. A different tube was used to obtain each pH value of every titration curve. The

Table 1. ^{1}H NMR chemical shifts of esters **2**, **3**, **6** and **8** and acids **4**, **7**, **9** and **10** at the pH obtained in 50 mM D_2O solutions

Compound	pH	H_2	H_4	H_5	CH	CH_2	OCH_2	OCH_3	CH_3
2	7.20	7.82(bs)	7.11(bs)	7.21(bs)		5.02(s)		3.80(s)	
3	7.68	7.87(bs)	7.10(bs)	7.33(bs)	a		4.35(m)		1.30(t)
6	8.80	7.80(bs)	7.04(bs)	7.27(bs)	5.12(m)	3.02(m)	4.09(q)		1.15(t)
8	7.92	7.83(bs)	7.08(bs)	7.26(bs)	5.57(t)	3.35(m)	4.27(q)		1.26(t)
							4.16(q)		1.20(t)
4	5.59	8.55(bs)	7.41(bs)	7.41(bs)		4.83(s)			
7	4.46	8.78(bs)	7.46(bs)	7.60(bs)	5.10(m)	2.83(m)			
9	4.25	8.86(bs)	7.47(bs)	7.58(bs)	5.38(dd)	3.29(m)	4.14(q)		1.19(t)
10[b]	3.29	8.84(bs)	7.47(bs)	7.57(bs)	5.35(dd)	3.21(m)			

Chemical shifts are expressed in ppm from internal TSP. Measurements were perfomed at 200.13 MHz (24 °C). Apparent multiplicity: s: singlet; bs: broad singlet; d: doublet; t: triplet; q: quartet; m: multiplet. a: D_2O exchange; b: obtained from reference 24.

Table 2. Apparent pK_a (pK'_a) values, titration limits (δ_1, δ_2), pH-titration ranges ($\Delta\delta$) in D_2O of the H-2 proton and $\log k'_o$

Compound	H-2 pK'_a[a]	δ_1	δ_2	$\Delta\delta$	$\log k'_0$
1	7.14	7.775	8.701	0.926	- 0.102
2	6.35[b]	7.706	8.819	1.113	- 0.126
3	5.02	7.860	9.069	1.209	
4	7.23 (7.13)	7.641 (7.520)	8.713 (8.860)	1.072 (1.340)	
6	6.45	7.799	8.972	1.173	0.866
7	7.37	7.705	8.772	1.067	
8	6.02	7.818	8.944	1.126	0.896
9	6.86	7.745	8.860	1.115	
10	7.32	7.696	8.799	1.103	

[a]Values in parentheses indicate results obtained in erythrocyte lysates.
[b]pK'_a of H-2 of compound **2** was previously reported as 7.10.[17] This value has been further refined in this work.

dependence of the H-2 chemical shift (δ) with respect to pH was obtained and computer fitted to the Henderson–Hasselbalch equation:

$$pH = pK'_a - \log [(\delta - \delta_1) / (\delta_2 - \delta)]$$

where δ_1 and δ_2 refer to the inferior (anionic) and superior (protonated) chemical shift limits of the H-2 titration curve. A three parameter, non-linear regression algorithm based on least squares minimization, allowed the determination of optimal values for δ_1, δ_2 and pK'_a. Observed changes in the chemical shift of the H-2 proton are derived from protonation/deprotonation at the imidazolic N-3 nitrogen atom. In addition, the pK'_a of **4** was also determined under similar titration conditions in erythrocyte lysates. Only a small difference was found between the pK'_a values determined in D_2O and in erythrocyte lysates. The latter value was used in the calculation of pH_i and pH_o in erythrocyte suspensions.

pK'_a Values of the H-2 proton in the acids **4**, **7**, **9** and **10** were always higher, than those of the corresponding esters **2**, **6** and **8**. In general pK'_a values of the esters were in the range 5.0–6.5, while those of the acids were in the range 6.9–7.4. Therefore, the acids present a more favorable pK'_a than the esters for the determination of pH in the physiological range. In all cases, the titration range of compounds **2–10** was significantly larger than that of **1**, providing, for the same change in pH, increased ^{1}H NMR resolution as compared to imidazole. Regarding the lipophilic character, an increase in hydrophobicity was determined for the esters in the order **2** < **1** < **6** < **8**. The corresponding acids showed negligible hydrophobicity, co-eluting with the potassium iodide used to determine the exclusion volume of the C_{18} reverse phase column (see experimental section).

Determination of intracellular pH (pH_i), extracellular pH (pH_o), and relative intracellular volume (RV_i)

Figure 1 shows ^{1}H NMR spectra from a representative erythrocyte suspension, before (1A) and after (1B) the addition of **2**. The ^{1}H NMR spectrum of rat erythrocytes (1A) is similar to previously described spectra from human erythocytes[21] (see Figure legend for resonance assignments). Shortly after the addition of **2**, only signals from **4** were detected (1B), indicating a rapid hydrolysis of the added ester. Complete hydrolysis is supported by the disappearance of the methoxy group of the ester (3.80 ppm), the appearance of a strong peak from methanol (3.25 ppm, resonance 8), and the reduction in the frequency difference between the H-4 and H-5 resonances (peak 11). It should be noted here that the chemical shift difference between the H-4 and H-5 resonances of the acid and the

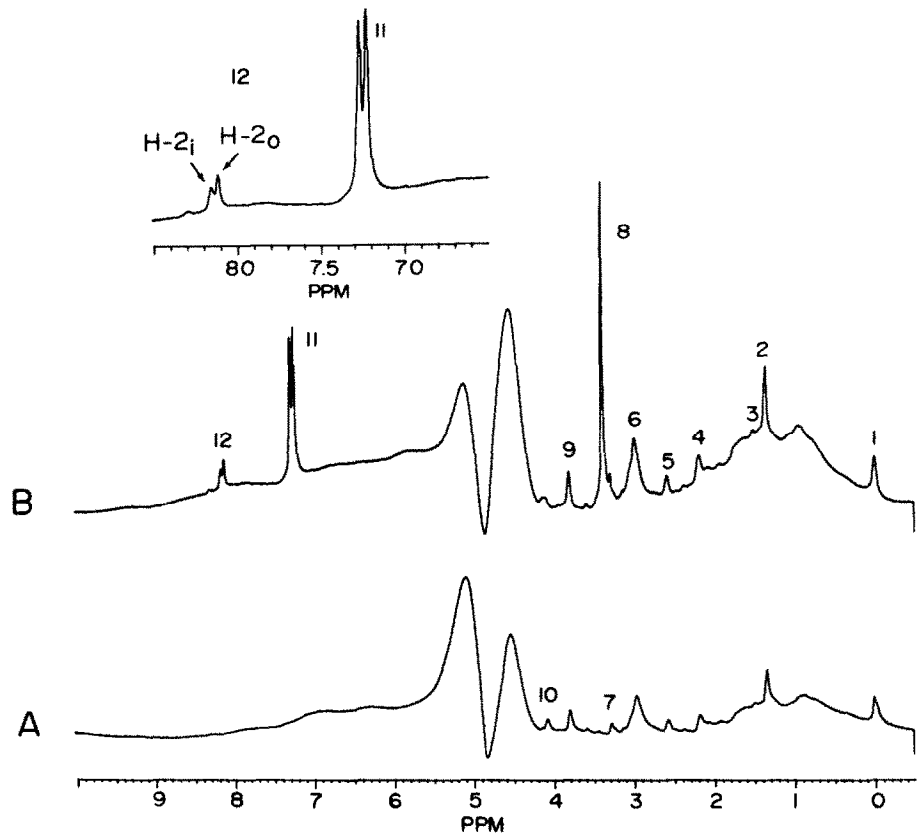


Figure 1. ^{1}H NMR spectra (360.1 MHz) of a representative erythrocyte preparation before (A) and after (B) the addition of 2 mM **2**. Acquisition conditions were as described in the experimental section. A 1 Hz artificial line broadening was applied prior to Fourier Transformation. 1: TSP, 2: lactate H-3, 3: alanine H-3, 4: glutamate H-4 in glutathione, 5: aspartate H-3,H-3', 6: CH_3 group of creatine, $(CH_3)_3N^+$ groups of choline, carnitine, and ergothioneine, 8: methanol, 9: H-2 from amino acids, 10: lactate H-2, 11: imidazolic H-4, H-5, 12: intracellular ($H\text{-}2_i$) and extracellular ($H\text{-}2_o$) imidazolic H-2.

ester were characteristic for every pH value, providing a convenient method to discriminate between these two compounds even when mixtures were present. Figure 1 shows that no other resonances from potential degradation products of **2** or **4** were detected. Very similar results to those shown in Figure 1, were obtained after the addition of **8**. However, regioselective hydrolysis of **8** to **9** only took place after 1 h incubation at 37 °C.

Notably, two signals from the H-2 proton of **4** (inset 1B) or **9** (not shown),[17] were consistently observed after the addition of **2** or **8** to erythrocyte suspensions. These two signals collapsed into a single H-2 resonance when the erythrocyte suspension was (i) solubilized with 1 % Triton X-100 or (ii) treated with 20 μg nigericin, a proton-ionophore which exchanges extracellular H^+ by intracellular K^+. These results indicate that the difference in chemical shift between the H-2 protons of **4** or **9**, is caused by the transmembrane pH gradient, the different H-2 signals being derived from molecules of **4** or **9** located in the intracellular (H-2_i) or extracellular (H-2_o) environments, respectively.

An example of the assignment of the H-2_i and H-2_o resonances of **4** is illustrated in Figure 2. In this experiment, the hematocrit was increased in the range 0.2–1.0 in different NMR tubes (panels A–E), maintaining constant at 2 mM the total amount of **2** added to every tube. A relative increase of the H-2 resonance at lower field (H-2_i) with increasing hematocrit was observed, indicating that the H-2_i resonance is derived from those molecules of **4** located in the intracellular space. pH_i and pH_o values were determined measuring the chemical shifts (δ_i and δ_o) of H-2_i and H-2_o protons, respectively. These values were substituted in the Henderson–Hasselbalch equation described above, using the corresponding figures for pK'_a, δ_1 and δ_2 previously determined in model solutions or in erythrocyte lysates (Table 2). Extracellular pH values were confirmed, after ^{1}H NMR spectroscopy, by determining the pH of the suspension using a combined glass electrode connected to a conventional pH meter. Both the intracellular and extracellular pH moved to more alkaline values with increasing hematocrit, as a result of the different initial values of pH in the erythrocyte pellet (pH = 7.30), and in the 2 mM solution of **4** (pH = 7.00). However, the transmembrane pH gradient, defined as the difference between the intracellular and extracellular pH, did not vary appreciably within the full hematocrit range, maintaining always an average value of 0.05 ± 0.01 pH units (acidic inside). This result indicates that the change in bulk magnetic susceptibility between the intracellular and extracellular spaces of erythrocyte preparations, a magnitude directly dependent on the hematocrit,[10,11] does not contribute appreciably to the transmembrane chemical shift difference between H-2_i and H-2_o protons of **4**. A different behavior, with transmembrane chemical shift differences depending on the hematocrit value, has been reported for other ^{31}P NMR probes of cellular volume.[10,11] In our ^{1}H NMR probes, the difference in pH between the intracellular and extracellular spaces seems to contribute almost exclusively to the transmembrane chemical shift difference between the H-2_i and H-2_o resonances.

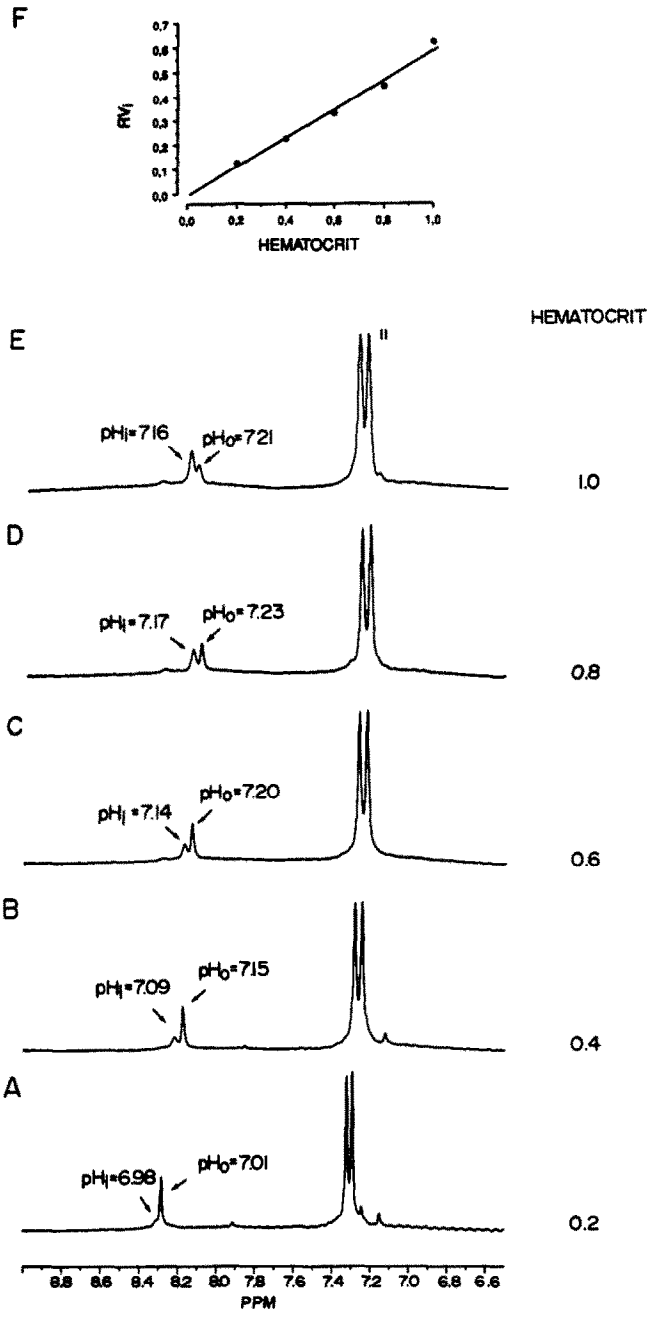


Figure 2. Effects of the increase in hematocrit on the relative intensities of H-2_i and H-2_o resonances of **4**. Acquisition conditions were as in Figure 1. Only the 6.5–9.0 ppm region of the ^{1}H NMR spectra is shown. The total incubation volume (500 μL) and added concentration of **2** (2 mM) were kept constant for increasing hematocrit values of: A: 0.2; B: 0.4; C: 0.6; D: 0.8; E: 1.0. pH_i, pH_o and relative intracellular volume (RV_i) were calculated as indicated in the experimetal section. F: relative intracellular volume (RV_i) and hematocrit value followed the linear relationship $RV_i = 0.58$ * hematocrit ($r^2 = 0.98$). Note the relative increase of the H-2_i resonance assigned to the intracellular space.

Figure 2 also shows that the relative volume of the intracellular and extracellular spaces can be determined from the relative areas of the H-2_i and H-2_o resonances of **4**. In particular, the relative intracellular volume (RV_i) can be calculated with the expression:

$$RV_i = \frac{\text{Area of H-2}_i}{\text{Area of H-2}_i + \text{Area of H-2}_o}$$

Both H-2_i and H-2_o resonances were integrated directly when complete resolution was observed. When partial overlapping appeared, individual H-2_i and H-2_o peaks were integrated after deconvolution of the composite resonance into the individual singlet components. The program PANIC (Parameter Adjustment in NMR by Iteration Calculation), a program from the BRUKER Library installed in our spectrometer, was used for this purpose. In addition, the absolute intracellular volume (AV_i) in the NMR tube can also be determined as:

$$AV_i = RV_i * \text{total sample volume}$$

These aspects are further illustrated in panel F, which summarizes the linear relationship obtained between the hematocrit value and the relative intracellular volume (RV_i). The RV_i of our erythrocyte preparations was 0.58 times their hematocrit value, which corresponds to a 580 µL total intracellular volume in a 1 mL suspension of packed erythrocytes.

Figure 3 illustrates the permeability properties of some relevant acids and esters. Panel A shows that the direct addition of **4** to an erythrocyte suspension originates distinct H-2_i and H-2_o signals, which are virtually identical to those obtained after the addition of ester **2** which is instantaneously hydrolyzed to **4** (panel B, *c.f.* Figure 1B). This result indicates that acid **4**, either added alone or derived from the hydrolysis of ester **2**, is permeable to the erythrocyte membrane. In contrast, addition of acid **9** (panel C), resulted only in one H-2_o resonance, the pH of which corresponded to the external pH (pH_o) determined potentiometrically. This finding indicated that **9** had not penetrated the erythrocyte interior. However, addition of ester **8** and incubation for 1 h at 37 °C (panel D), produced H-2_i and H-2_o signals from **9**, indicating that the ester **8** had entered the cellular interior and had been hydrolyzed to **9** in both, internal and external, compartments. Lack of incubation at 37 °C produced only incomplete hydrolysis of **8** in the extracellular environment (not shown). Ester **6** was not hydrolyzed nor penetrated the intracellular space and further experiments were not pursued.

A comparison of the permeability results obtained by ^1H NMR in erythrocyte preparations with the lipophilic character measured by HPLC (Table 2), indicates that there is no apparent relationship between these two properties. This is so because ester **8**, the most lipophilic compound, required 1 h incubation at 37 °C to cross the erythrocyte membrane. In contrast, acid **4**, a hydrophilic compound, readily distributed between inside and outside the cells immediately upon addition. Moreover, acid **9**, a hydrophilic but significantly larger molecule, did not pass through the membrane even after 1 h incubation at 37 °C. Therefore, the lipophilic character is not the only determinant of membrane permeability. Our results agree with previous work[10,11] which indicated that the diffusion of compounds through the erythrocyte membranes may be related also to molecular size and geometry.

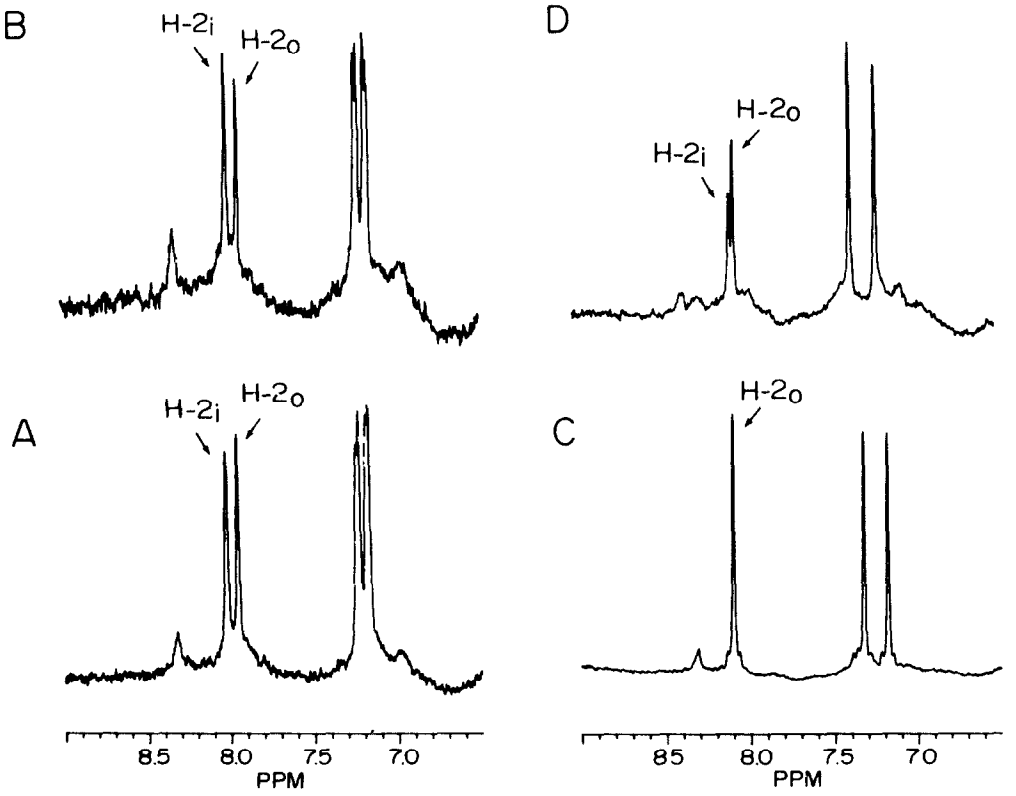


Figure 3. Comparison between the addition of acids **4** (A) and **9** (C) or esters **2** (B) and **8** (D) to erythrocyte suspensions. 1 µmol of the appropriate compound was added to erythrocyte suspensions (0.8 hematocrit) in a final volume of 0.5 mL. The spectrum shown in panel D was obtained after 1 h incubation at 37 °C. The remaining spectra were obtained, immediately after the addition of the probe.

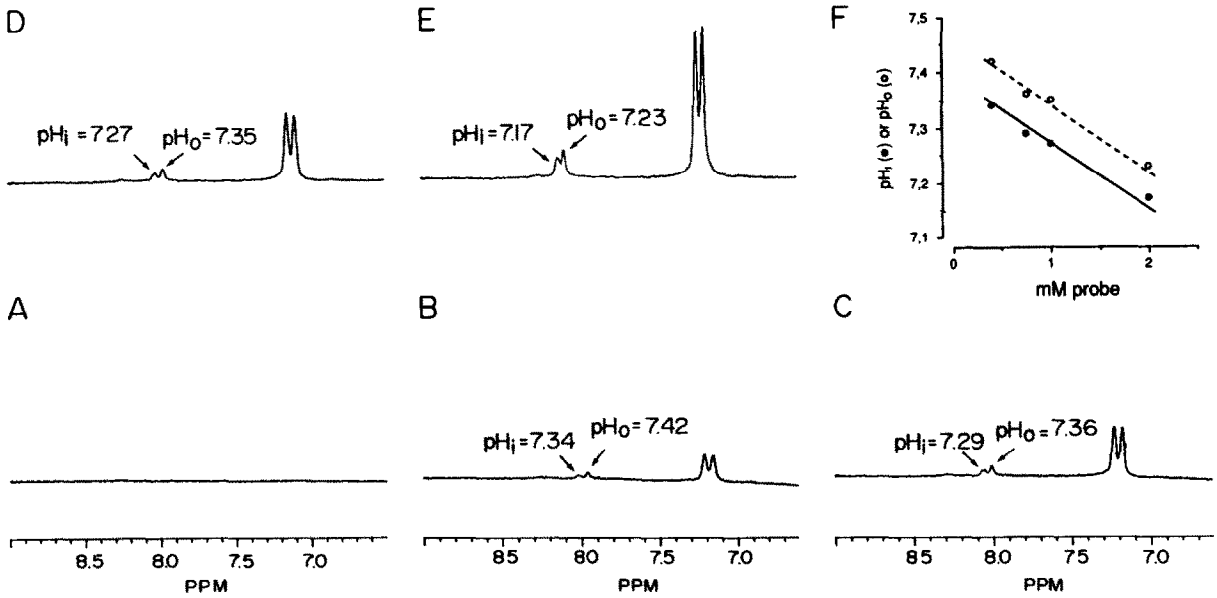


Figure 4. Effects of increasing concentrations of **4** on pH_i and pH_o as determined by ^1H NMR. ^1H NMR acquisition conditions were as in Figures 1 and 2. Erythrocyte suspensions (0.6 hematocrit) were incubated in a final volume of 500 µL with increasing concentrations of **4**. A: 0 mM, B: 0.4 mM, C: 0.7 mM, D: 1 mM and E: 2 mM. F: linear decrease of pH_i and pH_o for increasing concentrations of **4**. Note that pH_i and pH_o decrease in parallel.

Figure 4 depicts the effects of increasing concentrations of **4** up to 2 mM, on the pH_i and pH_o values of erythrocyte suspensions (panels A–E). Both H-2_o and H-2_i integrals increased in parallel and linearly with the concentration of **4**. This linear increase was maintained up to concentrations as high as 200 mM (not shown) with no apparent sign of saturation of the intracellular resonance with increasing concentrations of extracellular **4**. These results indicate that the transfer of **4** from the extracellular to the intracellular space proceeds by diffusion rather than through a saturable membrane transporter. As expected, increases in the concentration of added **4** resulted in a linear decrease of both pH_i and pH_o values. A decrease of 0.12 pH units/mM added **4** in both pH_i and pH_o was observed. Extrapolations to 0 mM added probe indicated an intrinsic pH_i value of 7.37 in the absence of **4** for this erythrocyte preparation. This perturbation of pH_i and pH_o upon addition of any extrinsic pH probe is unavoidable, but it can be minimized by decreasing the concentrations of added compound. Intra and extracellular resonances of **4** could be distinguished, after 2.5 min accumulation, for concentrations as low as 0.4 mM, and increased sensitivity would be expected for longer accumulation periods and higher magnetic fields.

Finally, Figure 5 (panels A–F) provides an illustrative example of the use of **4** in a study of the physiological interactions of erythrocyte volume, pH_i and pH_o. Erythrocytes prepared in hypotonic medium containing 100 mM NaCl (200 mOsM), were incubated in independent tubes with increasing concentrations of mannitol up to 400 mOsM, maintaining constant the hematocrit value (0.8), the final volume (500 µL) and the concentration of added **4** (2 mM). Increasing the osmolarity from 200 mOsM to 400 mOsM, resulted in (i) a reduction of the relative intracellular volume from 0.55 to 0.11 (panel H); (ii) a decrease in pH_o from 7.35 to 6.73 (panel G); (iii) no significant change in pH_i (panel G). The relative decrease of the intracellular H-2_i resonance as compared to the H-2_o resonance, observed in panels A–F, is consistent with the osmotically induced shrinking of the cells and the theory of the distribution of weak acids and bases between the extracellular and intracellular volumes.[6] This theory predicts that the concentration of the permeable acid **4** (or its non-ionized form of the carboxylic group) in the intra and extracellular spaces, is maintained constant during the osmotically induced decrease in intracellular volume. Therefore, cell volume reduction must be accompanied by an extrusion of intracellular **4** to the external medium, resulting in a relative decrease of the H-2_i area relative to H-2_o. The acidification detected in the extracellular space may involve an additional explanation. It is known that cells incubated in Na^+ containing solutions, react to hyperosmotic challenges, activating the Na^+/H^+ antiporter, a membrane transporter which catalyzes the electroneutral exchange of extracellular Na^+ by intracellular H^+.[22] The activation of this antiporter, increases the intracellular Na^+ concentration, favouring extracellular water to penetrate the erythrocyte interior, and contributing to regulate in this way the erythrocyte volume. As a corollary, the operation of the Na^+/H^+ antiporter, may result in the extrusion of H^+ which can contribute to the acidification of a non-buffered external medium.

Experimental Section

Chemistry

Melting points were obtained on a microscope hot stage and are uncorrected. NMR spectra were recorded on a Bruker AC200; ^1H (200.13 MHz) and ^{13}C (50.33 MHz). ^1H Chemical shifts are reported in ppm from internal tetramethylsilane (TMS) for $CDCl_3$ solutions and 3-trimethylsilyl(tetradeutero)propionic acid, sodium salt (TSP) for D_2O solutions. ^{13}C Chemical shifts in D_2O are reported in ppm from external dioxane. NMR data of esters

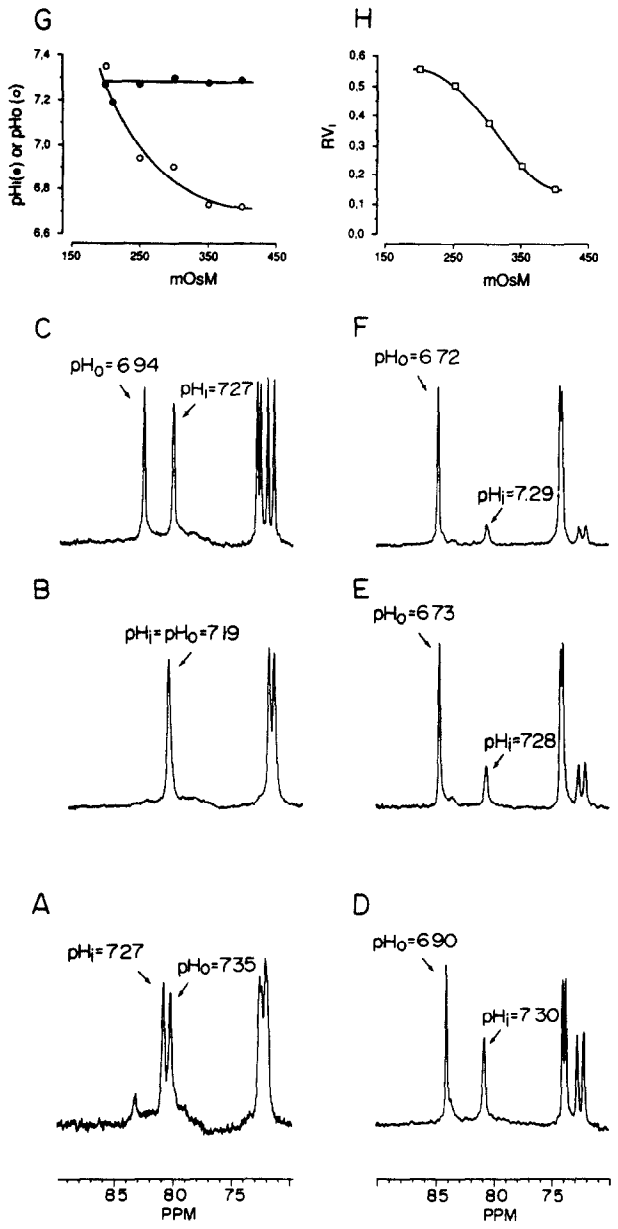


Figure 5. Effects of hypertonic treatment on pH_i, pH_o and RV_i. Acquisition and processing conditions were as in Figures 1 and 2. Erythrocytes prepared in 100 mM NaCl were incubated in a final volume of 535 µL with 2 mM **4** and increasing mannitol concentrations up to 400 mOsM. A: 200 mOsM, B: 210 mOsM, C: 250 mOsM, D: 300 mOsM, E: 350 mOsM and F: 400 mOsM. pH_i, pH_o, and RV_i were calculated as indicated in the experimental section. G: relationship between pH_i, pH_o and medium osmolarity, H: relationship between medium osmolarity and RV_i.

2, **3**, **6**, and **8** in $CDCl_3$ as well as the elemental analyses of picrates of **3**, **6**, and **8** have been previously reported by us.[17] The elemental analyses described here were carried out with a Perkin–Elmer 240 apparatus. Chromatographic purifications were performed at atmospheric pressure through columns using Merck silica gel 60 (70–230 mesh). Cationic exchange chromatography was performed on a Bio-Rad AG 50W-X8 resin using a 2N NH_4OH solution as eluent. HPLC was carried out with a Waters 600E multisolvent delivery system. Compounds were detected in the column effluent at 211 nm by a Waters 486 tunable absorbance detector. Retention data were collected by a Waters 746 data module. HPLC grade methanol was purchased from E. M. Merck Co. and reagent grade water was generated by a Millipore Milli-Q water purification system. Imidazole was obtained from Janssen Chimica. The rest of the reagents were obtained from Aldrich or Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Methyl imidazol-1-ylacetate **2**. To a stirred suspension of powdered potassium hydroxide (0.411 g, 7.35 mmol) and potassium carbonate (1 g, 7.35 mmol) in methylene chloride (20 mL) were added imidazole (0.5 g, 7.35 mmol) and 1,5-bis-(*N*-benzyl-*N*,*N*-diethylammonium)diethylether dichloride (BBDE Cl) (0.084 g, 0.18 mmol). To the vigorously stirred mixture, was added methyl bromoacetate (0.56 g, 3.67 mmol) and stirred for 3 h at rt. After filtering, the residue was washed with methylene chloride (2 x 15 mL) and the organic solutions were dried over anhydrous sodium sulfate. The methylene chloride was evaporated under vacuum and the residue purified over a silica gel column (9:1 CH_2Cl_2: ethanol) to yield **2** (0.414 g, 81 %), mp 54–55 °C (from hexane) Lit.[19C] oil. Compound **2** was stored in a dessicator over P_2O_5.

Diethyl imidazol-1-ylmalonate **3**. a) PTC alkylation method: to a stirred suspension of powdered potassium carbonate in methylene chloride (40 mL) were added imidazole (1 g, 14.7 mmol), 1,5-bis-(*N*-benzyl-*N*,*N*-diethylammonium)diethylether dichloride (BBDE Cl) (0.342 g, 0.73 mmol) and diethyl bromomalonate (3.51 g, 14.7 mmol). The mixture was vigorously stirred for 24 h at rt and filtered. The salts were washed with methylene chloride (2 x 30 mL) and the combined organic solutions were dried over anhydrous sodium sulfate. The methylene chloride was evaporated under vacuum and the residue was purified over a silica gel column (9:1 CH_2Cl_2: ethanol) to give **3** (1.64 g, 49 %). Kugelrohr distillation afforded pure **3**, $ot_{0.1}$ 165–200 °C; Picrate: mp 118–119 °C (from ethanol). Compound **3** was stored in a dessicator over P_2O_5. b) Acetonitrile/triethylamine method: to a solution of imidazole (0.5 g, 7.35 mmol) and dry triethylamine (0.74 g, 7.35 mmol) in dry acetonitrile (20 mL) was added diethyl bromomalonate (1.76 g, 7.35 mmol). The mixture was stirred at rt for 24 h, then filtered and the organic solvent was evaporated under vacuum. The residue was washed with water and extracted, several times, with methylene chloride. The organic extracts were dried over anhydrous sodium sulfate and then evaporated under vacuum. An oil was obtained which was purified as in a) to yield **3** (0.881 g, 53 %).

Ethyl imidazol-1-ylacetate **5**. Compound **3** was stored in a moist atmosphere at rt. After more than one month a solid appeared, which was treated with methylene chloride and compound **4** precipitated as a white solid which was filtered off. The organic solution was concentrated under vacuum and the residue purified through a silica gel column (90:10 CH_2Cl_2: ethanol) to separate the starting **3** and **5**. ^1H NMR ($CDCl_3$) δ: 1.27 (3H, t, J = 7.2 Hz, CH_3); 4.22 (2H, q, J = 7.2 Hz, OCH_2); 4.67 (2H, s, CH_2);

6.94 (1H, bs, H-5); 7.07 (1H, bs, H-4); 7.49 (1H, bs, H-2).

Diethyl 3-imidazol-1-ylglutarate **6**. A mixture of imidazole (0.5 mg, 7.35 mmol) and diethyl glutaconate (1.37 g, 7.35 mmol) was heated at 70 °C for 10 h. The reaction mixture was purified on a silica gel column (97:3 CH_2Cl_2: ethanol) and **6** (1.1 g, 59 %) was obtained as a yellow oil. Kugelrohr distillation afforded pure **6**, $ot_{0.1}$ 150–200 °C, mp 41–43 °C; Picrate: mp 111 °C (from ethanol).

Diethyl (±)-2-imidazol-1-ylsuccinate **8**. A mixture of imidazole (5 g, 73.53 mmol) and diethyl fumarate (12.65 g, 73.53 mmol) was heated at 100 °C for 24 h. Then the residue was purified over a silica gel column (98:2 CH_2Cl_2:ethanol) to give **8** (13.30 g, 75 %). Picrate: mp 95 °C (from ethanol). Compound **8** was stored in a desiccator over P_2O_5. This compound was also prepared in similar way from diethyl maleate. In this case compound **8** was obtained with 65 % yield.

Imidazol-1-ylacetic acid **4**. A solution of methyl imidazol-1-ylacetate **2** (0.5 g, 3.57 mmol) or diethyl imidazol-1-ylmalonate **3** (0.5 g, 2.21 mmol) in 20 mL of water was heated under reflux for 6 h. Then the solvent was evaporated under reduced pressure to give a hygroscopic residue. Treatment with ethanol gave a white solid, mp 257–258 °C (decomp.). Recrystallization from ethanol yielded the pure acid **4**, mp 267–269 °C (decomp.) Lit.[23] 269 °C.

3-Imidazol-1-ylglutaric acid **7**. Diethyl 3-imidazol-1-ylglutarate **5** (0.5 g, 1.96 mmol) was dissolved in 1 N aqueous hydrochloric acid (20 mL) and heated at 100 °C for 5 h. Then, the solvent was evaporated under reduced pressure to give a hygroscopic residue. This residue was chromatographed over cationic exchange resin to give pure **7**, then recrystallized from 90 % ethanol and dried over P_2O_5 at 80 °C/0.1 mmHg, mp 181–183 °C. ^1H NMR (D_2O) δ: 2.91–2.99 (4H, m, 2 x CH_2); 5.15–5.23 (1H, m, CH); 7.49 (1H, bs, H-4); 7.66 (1H, bs, H-5); 8.87 (1H, bs, H-2); ^{13}C NMR (D_2O) δ: 41.1 (t, CH_2); 56.1 (d, CH); 120.4 (d, C-5); 120.8 (d, C-4); 175.9 (s, COOH). Anal. Calcd for $C_8H_{12}N_2O_4$ $1/2H_2O$: C, 46.37; H, 5.35, N,13.52; Found: C, 46.82; H, 4.85; N, 13.31.

(±)-2-Imidazol-1-yl-3-ethoxycarbonylpropionic acid **9**. Diethyl 2-imidazol-1-ylsuccinate **8** (0.5 g, 2.08 mmol) was dissolved in water (20 mL) and heated at 100 °C for 18 h. Then the solvent was evaporated under reduced pressure to give a hygroscopic residue. Treatment with ethanol gave a crystalline white product which was recrystallised from ethanol and dried over P_2O_5 at 80 °C/0.1 mmHg, mp 141–143 °C. Anal. Calcd for $C_9H_{12}N_2O_4.1/4H_2O$: C, 49.68; H, 5.67; N, 12.87; Found: C, 49.63; H, 5.50; N, 12.93.

(±)-2-Imidazol-1-ylsuccinic acid **10** *HCl*. Diethyl 2-imidazol-1-ylsuccinate **8** (0.5 g, 2.08 mmol) was dissolved in 1 N aqueous hydrochloric acid (20 mL) and heated at 100 °C for 6 h. Then, the solvent was evaporated under reduced pressure to give a hygroscopic residue. Treatment with ethanol gave a white solid mp 249–250 °C (decomp.) ^1H NMR data[17] were in agreement with the hydrochloride form of the previously described **10**.[24]

Lipophilic character

It is well known that the lipophilic character is directly related to the octanol/water partition coefficient (P). However, the direct determination of P by the conventional shaking-flask method[25] is cumbersome. The high-performance liquid chromatography (HPLC) method provides a convenient alternative. This method allows the determination of log k'_o, a parameter directly related to the lipophilic character. In most cases, values of log k'_o increased linearly with log P.[26] We have determined the capacity factors (k') of compounds **1**, **2**, **6** and **8** by reverse phase HPLC (25 °C), using a Nova-Pak C_{18} column (8 mm x 100 mm Cartridge). Compounds were dissolved in methanol at a concentration of 1 μg/mL and injected separately. The mobile phase consisted of mixtures of MeOH/H_2O solutions at 65 %, 70 %, 75 %, 85 % and 100 % (v/v), respectively, which were run in the isocratic mode (1 mL/min flow rate). Retention times for the different elution conditions were determined by continuous monitoring of the absorbance of the effluent at 211 nm . The results are the mean of three independent runs for every compound. Capacity factors (k') were determined from a comparison of the observed retention time for the appropriate compound (t_r) with the elution time (t_o) of unretained potassium iodide. The following equation was used:

$$k' = \frac{t_r - t_o}{t_o}$$

Potassium iodide was confirmed not to be retained on the stationary phase and showed an identical retention time in all mobile phases. k' Values correlate linearly with the organic solvent concentration in the eluent following the equation[27]:

$$\log k' = \log k'_o + b\,C$$

where log k'_o is the value of log k' at zero methanol concentration, b is the slope of the straight line and C is the methanol concentration (%, v/v).

Biological evaluation

Preparation and characterization of erythrocytes, incubation conditions and experimental design. Erythrocytes were prepared from the blood of adult male Sprague–Dawley rats fed *ad libitum*. Whole blood, taken from the inferior caval vein, was centrifuged at 3000 *g* and washed three times with isotonic saline solution (155 mM NaCl). The erythrocyte pellet obtained after the last wash was resuspended in 155 mM NaCl (20 % D_2O) and concentrated to an hematocrit value of 1.0 prior to use. Under these conditions the erythrocyte pellets had (i) a protein concentration of 67.4 ± 4.2 mg /mL as determined by the biuret method; (ii) a density of 1.074 g/mL, as measured by centrifugation in a Percoll/155 mM NaCl density gradient; (iii) a wet weight/dry weight ratio of 3.3 ± 0.04.

Average erythrocyte diameter was 9.5 ± 0.8 μ, as determined by computerized video microscopy. The erythrocyte suspension was placed in 5 mm NMR tubes at the indicated hematocrit and compounds **1**, **2**, **4**, **6**, **8**, and **9** were added to a final concentration of 0.4–4 mM in a total incubation volume of 0.5 mL. Routinely, ^{1}H NMR spectra were taken (22 °C) before and inmediately after the addition of the probe. Erythrocyte lysates were prepared by ultrasonic disruption (MSE Soniprep 150, 23 KHz, 5 min, 4 °C).

^{1}H NMR spectroscopy of erythrocyte suspensions. ^{1}H NMR spectra of erythrocyte suspensions were obtained at 360.13 MHz in a Bruker AM-360 spectrometer using a commercial 5 mm NMR probe. A single-pulse presaturation sequence was routinely used. Acquisition conditions were: 90 ° pulses, 3968 Hz spectral width, 16 K computer memory (1.3 s acquisition time), 2.3 s total cycle time and 64 transients. A 1 s presaturating pulse was applied with the decoupler before the 90 ° observing pulse. Therefore, the total time resolution of ^{1}H NMR spectra was 147 s. ^{1}H NMR assignments of erythrocyte resonances were made by comparison with literature values and confirmed where appropriate by the addition of the authentic compounds. Chemical shifts were referenced to internal TSP.

Acknowledgements

This work was supported in part by grants of the Community of Madrid (C015/91), the Spanish D.G.C.Y.T (PM-89-010 and PM-92-011) and the Rodriguez–Pascual Foundation to S. C. and P. B. M. G. received a postdoctoral fellowship from the Community of Madrid. P. Z. and F. C. were supported by fellowships from the Spanish Ministry of Education and Science. The expert technical assistance of Mrs B. de Mateo and Mrs R. Albadalejo is gratefully acknowledged. The catalyst BBDE Cl was a generous gift of Dr J. Álvarez-Builla.

References and Notes

1. Roos, A.; Boron, W. F. *Physiological Reviews* **1981**, *61*, 296.

2. Henderson, R. M.; Graf, J. *pH Homeostasis: Mechanisms and Control*, pp 5–26, Haussinger, D., Ed.; Academic Press: New York, 1988.

3. Busa, W. B. *Ann. Rev. Physiol.* **1986**, *48*, 389.

4. Moolenaar, N. H. *Ann. Rev. Physiol.* **1986**, *48*, 363.

5. Haüssinger, D.; Lang, F. *Biochim. Biophys. Acta* **1991**, *1071*, 331.

6. Cohen, R. D.; Iles, R. A. *C.R.C. Crit. Revs Clin. Lab. Chem.* **1975**, *6*, 101.

7. Nuccitelli, R. *Intracellular pH: its Measurements, Regulation, and Utilization in Cellular Functions*, pp 161–169, Nuccitelli, R.; Deamer, D. W. Eds.; NewYork: Liss, 1982.

8. Tsien, R. Y. *Methods in Cell Biology* **1989**, *30*, 127.

9. Gadian, D. G. *Nuclear Magnetic Resonance and its Applications to Living Systems*, pp 29–35, Oxford University Press: Oxford 1982.

10. Kirk, K.; Kuchell, P. W. *Biochemistry* **1988**, *27*, 8795.

11. Kirk, K.; Kuchell, P. W. *Biochemistry* **1988**, *27*, 8803.

12. Deutsch, C.; Taylor, J. S.; Wilson, D. F. *Proc. Natl Acad. Sci. U.S.A.* **1982**, *79*, 7944.

13. Arús, C.; Yen-Chung, C.; Barany, M. *Biochim. Biophys. Acta* **1985**, *844*, 91.

14. (a) Xu, A. S. L.; Waldeck, A. R.; Kuchel, P. W. *NMR in Biomed.* **1993**, *6*, 136; (b) Adebodun, F.; Post, J. F. M. *NMR in Biomed.* **1993**, *6*, 125.

15. Yoshizaki, K.; Seo, Y.; Nishikawa, H. *Biochim. Biophys. Acta* **1981**, *678*, 283.

16. Rabenstein, D. L.; Isab, A. *Anal. Biochem.* **1982**, *121*, 423.

17. Gil, M. S.; Cruz, F.; Cerdan, S.; Ballesteros, P. *BioMed. Chem. Lett.* **1992**, *2*, 171.

18. Alvarez-Builla, J.; Vaquero, J. J.; Navio, J .L.; Cabello, J. F.; Sunkel, C.; Fau de Casa-Juana, M.; Dorrego, F.; Santos, L. *Tetrahedron* **1990**, *46*, 967.

19. (a) Pellicciari, R.; Curini, M.; Spagnoli, N.; Ceccherelli, P. *Synthesis* **1981**, 629; (b) Kamijo, T.; Yamamoto, R.; Harada, H.; Iizuka, K. *Chem. Pharm. Bull.* **1983**, *31*,1213; (c) Sunjic, V.; Kajfez, F.; Milder, P. *Croat. Chem. Acta* **1969**, *41*,107.

20. Iselin, B. M.; Hoffmann, K. *J. Am. Chem. Soc.* **1954**, *76*, 3220.

21. Rabenstein, D. L.; Millis, K. K.; Strauss, E. J. *Anal. Chem.* **1988**, *60*, 1380A.

22. Grinstein, S.; Clark, C. A.; Rodstein, A. *J. Gen. Physiol.* **1983**, *82*, 619.

23. Birkofer, L.; Righter, P.; Ritter, A. *Chem. Ber.* **1960**, *93*, 2804.

24. Mroczkiewicz, A. *Acta Pol. Pharm.* **1984**, *41*, 435.

25. Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525.

26. Miyake, K.; Mizumo, N.; Terada, H. *Chem. Pharm. Bull.* **1986**, 34, 4787.

27. Hulshoff, A.; Perrin, J. H. *J. Chromatogr.* **1976**, *129*, 263.

(*Received* 1 *October* 1993; *accepted* 6 *April* 1994)