# Augmented Water Binding and Low Cellular Water Content in Erythrocytes of Camel and Camelids

Péter Bogner,* Péter Csutora,* Ivan L. Cameron,[#] Denys N. Wheatley,[§] and Attila Miseta*

*Department of Clinical Chemistry, University Medical School at Pécs, Pécs, Ifjúság u. 13. 7643, Hungary; [#]Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7762 USA; and [§]Department of Pathology, The University of Aberdeen, AB9 2ZD Aberdeen, Scotland

ABSTRACT   We investigated a link between hemoglobin primary structure, hemoglobin hydrophobicity-hydrophilicity, and erythrocyte water content in various mammalian species. Some hemoglobin molecules, particularly those of the camel and camelids, contain more charged amino acid residues and are more hydrophilic than the hemoglobins of human and a number of other mammalian species. To test the in vivo significance of these alterations of hemoglobin primary structure, we determined the osmotically unresponsive erythrocyte water fractions in mannit solutions of various osmolarities at 4°C. Among the species investigated, the size of the osmotically unresponsive erythrocyte water fraction relates in a positive linear way to hemoglobin hydrophilicity. The extreme low total erythrocyte water content of camel erythrocytes (1.1–1.3 g water/g dry mass) may be explained by a comparatively high osmotically unresponsive erythrocyte water fraction. It is proposed that alterations of hemoglobin sequences of camel and camelids may be the part of a natural selection process aimed at protecting these animals against osmotic dehydration in arid environments.

## INTRODUCTION

Water-protein interactions likely play an important role in shaping the cellular water content, yet the test of this hypothesis is complex.

This complexity is due to several factors: 1) There is no consensus about the water binding characteristics of individual proteins. Nevertheless, a number of protein hydrophobicity-hydrophilicity prediction methods are widely used to predict membrane-spanning protein domains, protein antigenicity, or the overall hydrophobicity-hydrophilicity properties of proteins (Bull and Breeze, 1974; Janin, 1979; Hopp and Woods, 1981; Guy, 1985; Radzicka and Wolfenden, 1988; Cowan and Whittaker, 1990). The solution nonideality characteristics of individual amino acids and peptides have to be resolved before a better, perhaps quantitative prediction of protein-water interactions based on constituent amino acids can be recommended (Zimmerman et al., 1993; Keener et al., 1995). 2) Most cell types contain a large variety of proteins, which, owing to their diverse amino acid compositions, interact differently with water. There is no consensus about the extent and significance of perturbed (nonbulklike) water within cells (Clegg, 1984; Wheatley, 1991; Cameron et al., 1997). Conveniently, most researchers contemplate that all of the cell water has solvent properties similar to that seen in dilute aqueous solutions. 3) The interactions of nonprotein macromolecules, like nucleic acids or polysaccharides, contribute significantly toward water binding within the cell. 4)

Systemic alterations of the cellular environment, which are known to induce conformation changes in proteins (i.e., pH, ionic strength), cause changes not only in protein hydration, but also in ion/molecule permeabilities of cells.

However, recent progress in medical and veterinary genetics allowed us to attempt to substantiate a link between hemoglobin hydration and erythrocyte volumes, while largely bypassing the above-mentioned theoretical and experimental problems.

Our choice experimental cell type, the mature mammalian erythrocyte, is highly differentiated. Mature erythrocytes lack cellular organelles and are composed primarily of water and proteins. Their water content varies between 1.1 and 2.4 g water/g dry mass (52–74% water) in different species but is remarkably constant among healthy individuals of the same species (68–70% in human).

Importantly, more than 95% of the mature erythrocyte protein content is hemoglobin, and the remaining less than 5% represents the enzymes of the Embden-Meyerhof pathway, pentose phosphate shunt, and the proteins of the plasma membrane/subplasma membrane cytoskeleton.

Our working hypothesis was that owing to the predominance of hemoglobin among erythrocyte proteins, alterations of hemoglobin amino acid sequences may influence hemoglobin-water interactions and erythrocyte water concentrations.

The amino acid sequences and the hydrophobicity-hydrophilicity profiles of hemoglobins of 16 mammalian species were compared. We show 1) that there are characteristic differences between both the hemoglobin $\alpha$ (HBA) and $\beta$ (HBB) chain sequences of different animals, 2) and that these nonconservative amino acid replacements alter the water-binding capabilities of hemoglobins and 3) the osmotic properties of erythrocytes, and 4) are likely to contribute to the apparent water content of erythrocytes.

## MATERIALS AND METHODS

### Blood samples

Human blood samples were obtained from the cubital veins of young healthy volunteers. Blood samples from camel, rat, mice, rabbit, golden hamster, guinea pig, horse, goat, sheep, bovine, swine, dog, cat, rhesus monkey, and Arabian baboon were drawn from a suitable vein of the animal. After the blood was drawn into test tubes containing 100 IU heparin/ml blood, they were turned upside down and back five times to mix the blood with heparin. Thereafter blood samples were shifted to wet ice and used for experimental analysis within ~90 min. We obtained blood samples from rat, mice, rabbit, and golden hamster in the university animal facility. Blood samples from horse, goat, sheep, bovine, and swine were drawn at the Department of Animal Breeding, Pannon University of Agriculture, Kaposvar. Blood from dogs was obtained at the Department of Experimental Surgery. Blood from cats and rhesus monkeys was drawn at the Department of Physiology. Blood from camels and Arabian baboons was drawn at the city zoo. All of these blood collections took place in our presence, apart from the ones that took place at the Department of Physiology (cat and rhesus monkey) and were carried out according to our instructions.

### Determination of erythrocyte water, potassium, and sodium contents

Heparinized blood samples were first pelleted with a tabletop centrifuge (Hettich, EBA 3S) at $3000 \times g$ for 10 min at 4°C, the supernatants and buffy coats were carefully removed, and 0.5–1.0-ml aliquots of the pelleted erythrocytes were transferred into microcentrifuge tubes of known weight. The samples were pelleted again at $15,000 \times g$ for 10 min at 4°C, and the supernatants were removed. The samples were centrifuged for an additional 5 min as described above, and any trace of residual water was removed carefully with a 20-$\mu$l pipetteman. The wet weights of samples were measured with an analytical balance (OWA Labor, Oschatz, Germany) to an accuracy of 0.1 mg.

The pelleted erythrocyte samples were dried in a Savant SC-110 speed vacuum system, with the heating set at "high" until no further loss of weight occurred (usually ~3–4 h), and the dry weight of the samples was measured.

The net wet and dry weights of the sample equal the wet weight and dry weight minus the microcentrifuge tube weight, respectively. The difference between the net wet weight and net dry weight equals the mass of water evaporated during the drying procedure. Based on these data, the water contents were calculated and expressed either as the percentage of the total erythrocyte mass or as g water/g dry mass. The trapped water was 2.2–3.6% for the various erythrocytes investigated, as determined by a trypan blue exclusion test.

For $K^+$ and $Na^+$ concentration measurements, the dried erythrocyte samples were treated with 0.6–1 ml 1 M HCl at room temperature for 24 h. The samples were thoroughly vortexed after the addition of HCl and pelleted by centrifugation at $15,000 \times g$ for 5 min at the end of the incubation. The supernatants were transferred into new microcentrifuge tubes, and the $K^+$ and $Na^+$ concentrations were measured with an Eppendorf EFOX 5070 flame photometer. Cellular $K^+$ and $Na^+$ concentrations were calculated by considering dilution factors and the sample water contents.

### Osmotic tests

A method introduced by Ling and Negendank was modified for our experimental goals (Ling and Negendank, 1970). Briefly, erythrocyte samples (0.5 g) were incubated in mannit solutions of various osmolarities (150–500 mOsm) at 1:10 erythrocyte:media volume ratio, at 4°C for 3 h. Because the osmolarity of the bathing solutions is altered slightly by electrolytes leaking from the erythrocytes, we drew aliquots and checked the osmolarity of each sample with a Vescor (vapor pressure) osmometer

at the end of the experiment. Cells were harvested by centrifugation, and their water content was determined as described above.

### Amino acid sequences of hemoglobin molecules and sequence comparisons

All sequences shown in the present report were obtained from the SwissProt protein sequence database. Note that some sequences are identical in two or more species. The HBA sequences (with accession number) were: AC:P01922, human, chimpanzee, and pygmy chimpanzee (bonobo); AC:P01925, rhesus macaque and japanese macaque; AC:P01952, dog and coyote; AC:P07405, cat; AC:P01958, horse; AC:P01948, rabbit; AC:P01945, golden hamster; AC:P01946, rat; AC:P01966, bovine; AC:P01974, Arabian camel (dromedary) and bactrian camel; AC:P01970, goat, barbary sheep (aoudad), and sheep; AC:P01965, swine; AC:P01931, yellow baboon and olive baboon; AC:P01973, llama; AC:P01947, guinea pig.

The HBB sequences were: AC:P02023, human, chimpanzee, and pygmy chimpanzee; AC:02070, bovine; AC:P02069, Arabian camel and bactrian camel; AC:P02062, horse; AC:P02056, dog and coyote; AC:P07412, cat; AC:P02077, goat; AC:P02075, sheep; AC:P02057, rabbit; AC:P02026, rhesus macaque; AC:P02094, golden hamster; AC:P02091, rat; AC:P02067, swine; AC:P02030, yellow baboon; AC:P02095, guinea pig; AC:P02068, llama, vicuña, alpaca, and guanaco.

Amino acid sequence alignments were determined according to the method developed by Myers and Miller (1988). We used a structure genetic matrix based on the work of Feng et al. (1985), with an open gap cost = 5 and unit gap cost = 5. Multiple amino acid sequence alignments were made with the "Clustal" program (Higgins and Sharp, 1988, 1989).

### Calculating and plotting of hydrophobicity-hydrophilicity profiles

We used the amino acid hydrophobicity-hydrophilicity rankings of several authors to predict hemoglobin hydrophobicities (Hopp and Woods, 1981; Guy, 1985; Radzicka and Wolfenden, 1988; Cowan and Whittaker, 1990). Most of our calculations and all figures shown in the present report are based on the ranking of Cowan and Whittaker (1990).

A window of five amino acids was used for calculation of protein hydrophobicity-hydrophilicity profiles. We used an exponential weight variation model, with the weights at the edges set at 20%.

Human and camel HBA and HBB sequences were plotted in an *xy* graph. The *x* axis indicates the amino acid number in the sequence. The *y* axis represents the relative hydrophilicity of the corresponding regions of human and camel HBA and HBB sequences.

Sometimes as an arbitrary measure of protein hydrophobicity/hydrophilicity, we summed the scores of each amino acid residue in a given HBA or HBB chain. These scores were used to compare the overall hydrophobicities of hemoglobins of various species.

## RESULTS

### Amino acid sequence alignments and amino acid compositions of hemoglobins of various mammalian species

We selected 16 mammalian hemoglobin $\alpha$ and $\beta$ chain sequences. The selection was governed by the availability of relevant amino acid sequence information in the protein sequence database and the availability of the animal species for experimental purpose (see Materials and Methods).

Hemoglobins of seven mammalian species representing the primate (human), carnivora (dog), perissodactyla (horse), artiodactyla (pig, camel, bovine), and rodentia (rab-

```
HBA        (141 amino acids)
human      VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVA
horse      VLSAADKTNVKAAWSKVGGHAGEYGAEALERMFLGFPTTKTYFPHFDLSHGSAQVKAHGKKVGDALTLAVG
bovine     VLSAADKGNVKAAWGKVGGHAAEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVE
swine      VLSAADKANVKAAWGKVGGQAGAHGAEALERMFLGFPTTKTYFPHFNLSHGSDQVKAHGQKVADALTKAVG
camel      VLSSKDKTNVKTAFGKIGGHAAEYGAEALERMFLGFPTTKTYFPHFDLSHGSAQVKAHGKKVGDALTKAAD
rabbit     VLSPADKTNIKTAWEKIGSHGGEYGAEAVERMFLGFPTTKTYFPHFDFTHGSEQIKAHGKKVSEALTKAVG
dog        VLSPADKTNIKSTWDKIGGHAGDYGGEALDRTFQSFPTTKTYFPHFDLSPGSAQVKAHGKKVADALTTAVA
           ***. **.*.*.....*.*.......*.**..* * .***********...**.*.*.** **..****..
human      HVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR
horse      HLDDLPGALSNLSDLHAHKLRVDPVNFKLLSHCLLSTLAVHLPNDFTPAVHASLDKFLSSVSTVLTSKYR
bovine     HLDDLPGALSELSDLHAHKLRVDPVNFKLLSHSLLVTLASHLPSDFTPAVHASLDKFLANVSTVLTSKYR
swine      HLDDLPGALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHHPDDFNPSVHASLDKFLANVSTVLTSKYR
camel      HLDDLPSALSALSDLHAHKLRVDPVNFKLLSHCLLVTVAAHHPGDFTPSVHASLDKFLANVSTVLTSKYR
rabbit     HLDDLPGALSTLSDLHAHKLRVDPVNFKLLSHCLLVTLANHHPSEFTPAVHASLDKFLANVSTVLTSKYR
dog        HLDDLPGALSALSDLHAYKLRVDPVNFKLLSHCLLVTLACHHPTEFTPAVHASLDKFFAAVSTVLTSKYR
           ****.*.***.******.*************.** *.* * *..*.*.*.*******...*********
```

```
HBB        (144 – 145 amino acids)
human      VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSD
horse      VQLSGEEKAAVLALWDKVNEEEVGGEALGRLLVVYPWTQRFFDSFGDLSNPGAVMGNPKVKAHGKKVLHSFGE
bovine     M-LTAEEKAAVTAFWGKVKVDEVGGEALGRLLVVYPWTQRFFESFGDLSTADAVMNNPKVKAHGKKVLDSFSN
swine      VHLSAEEKEAVLGLWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSNADAVMGNPKVKAHGKKVLQSFSD
camel      VHLSGDEKNAVHGLWSKVKVDEVGGEALGRLLVVYPWTRRFFESFGDLSTADAVMNNPKVKAHGSKVLNSFGD
rabbit     VHLSSEEKSAVTALWGKVNVEEVGGEALGRLLVVYPWTQRFFESFGDLSSANAVMNNPKVKAHGKKVLAAFSE
dog        VHLTAEEKSLVSGLWGKVNVDEVGGEALGRLLIVYPWTQRFFDSFGDLSTPDAVMSNAKVKAHGKKVLNSFSD
           . *. .**. * ..*.**. .**********.****.***.******. ..***.*.*****.*** .*.
human      GLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH
horse      GVHHLDNLKGTFAALSELHCDKLHVDPENFRLLGNVLVVVLARHFGKDFTPELQASYQKVVAGVANALAHKYH
bovine     GMKHLDDLKGTFAALSELHCDKLHVDPENFKLLGNVLVVVLARNFGKEFTPVLQADFQKVVAGVANALAHRYH
swine      GLKHLDNLKGTFAKLSELHCDQLHVDPENFRLLGNVIVVVLARRLGHDFNPDVQAAFQKVVAGVANALAHKYH
camel      GLNHLDNLKGTYAKLSELHCDKLHVDPENFRLLGNVLVVVLARHFGKEFTPDLQAAYQKVVAGVANALAHRYH
rabbit     GLSHLDNLKGTFAKLSELHCDKLHVDPENFRLLGNVLVIVLSHHFGKEFTPQVQAAYQKVVAGVANALAHKYH
dog        GLKNLDNLKGTFAKLSELHCDKLHVDPENFKLLGNVLVCVLAHHFGKEFTPQVQAAYQKVVAGVANALAHKYH
           *. .**.****.* *******.********.*****.* **....*..*.* .**..*************.**
```

FIGURE 1 Amino acid sequence alignments of human, horse, bovine, swine, camel, rabbit, and dog hemoglobin α chain (HBA) and hemoglobin β chain (HBB) sequences (Myers and Miller, 1988). A structure genetic matrix was used (Feng et al., 1985). Amino acids said to be similar are A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W. Stars (*) indicate identical residues; dots (.) label similar residues.

bit) orders retain a remarkable degree of homology. An overall comparison of the amino acid sequences of HBA and HBB subunits of these species resulted in 63.8% and 66.4% identities and 30.5% and 26.7% similarities (replacements with physicochemically similar amino acids), respectively (Fig. 1). Identities and similarities for the HBA and HBB chains of all 16 species investigated were 50.4%, 51.4% and 36.2%, 36.3%, respectively (alignment not shown).

Owing to this remarkable evolutionary conservation of hemoglobin sequences, only ~10–13% of the amino acid residues (~14–17 amino acid residues per HBA or HBB chain) may differ in their physicochemical properties.

Nevertheless, the comparative amino acid composition table of these hemoglobins shows characteristic differences (Table 1). Most notably, the number of positively (Arg, Lys, His) and negatively (Asp, Glu) charged amino acid residues is highest in the camel, followed by its relative the llama, among the mammalian species investigated.

We note that the HBA and HBB sequences of the one-humped (*Camelus dromedarius*) and two-humped (*Camelus bactrianus*) camels are identical. The HBB sequences of llama (*Lama glama*), vicuña (*Lama vicuna*), alpaca (*Lama guanicoe pecos*), and guanaco (*Lama guanicoe*) are identical. Changes in their HBA chains are restricted to a few conservative amino acid substitutions (data not shown).

## Hydrophobicity-hydrophilicity profiles of HBA and HBB chains of human and camel

The hydrophobicity-hydrophilicity profiles of proteins are based on their amino acid sequences and the hydrophobic-

ity-hydrophilicity properties of individual amino acids. However, a number of different hydrophobicity-hydrophilicity rankings of amino acids are available. This is due mainly to the different results of various methods used for the determination of amino acid hydrophilicity. For this reason, we show four hydrophobicity-hydrophilicity ranking profiles (Hopp and Woods, 1981; Guy, 1985; Radzicka and Wolfenden, 1988; Cowan and Whittaker, 1990) (Table 2). The hydrophobicity-hydrophilicity scores for each HBA and HBB amino acid residue were combined for each species. Higher scores may correspond to increased hydrophobicity (Radzicka and Wolfenden, 1988; Cowan and Whit-

**TABLE 1   Amino acid compositions of various hemoglobins**

| | Hydrophilic (Charged) | | | Intermediate | Hydrophobic |
|---|---|---|---|---|---|
| | D,E | H,K,R | Total | A,G,P,S,T,C,N,Q | I,L,V,M,F,Y,W |
| *Camel* | 58 | 106 | 164 | 224 | 186 |
| Human | 54 | 94 | 148 | 238 | 188 |
| Bovine | 60 | 94 | 154 | 226 | 192 |
| Swine | 58 | 98 | 156 | 230 | 190 |
| Rabbit | 56 | 100 | 156 | 232 | 188 |
| *Llama* | 60 | 102 | 162 | 226 | 186 |
| Rhesus | 54 | 94 | 148 | 236 | 190 |
| Baboon | 58 | 100 | 158 | 226 | 190 |
| Goat | 58 | 96 | 154 | 228 | 190 |
| Sheep | 58 | 96 | 154 | 230 | 190 |
| Horse | 58 | 96 | 154 | 228 | 192 |
| Dog | 56 | 94 | 150 | 238 | 186 |
| Cat | 56 | 92 | 148 | 240 | 186 |
| Rat | 56 | 100 | 156 | 238 | 180 |
| Hamster | 54 | 100 | 154 | 232 | 188 |
| Guinea pig | 52 | 98 | 150 | 240 | 184 |

**TABLE 2   Predicted hydrophilicities of various hemoglobins**

| | Cowan | | Guy | | Hopp | | Radzicka | |
|---|---|---|---|---|---|---|---|---|
| | Human | 157.53 | Cat | 138.22 | Guinea pig | 142.05 | Human | 198.48 |
| | Rhesus | 156.79 | Guinea pig | 138.65 | Human | 142.54 | Rhesus | 198.13 |
| | Guinea pig | 156.09 | Hamster | 138.98 | Hamster | 142.73 | Hamster | 196.93 |
| | Hamster | 155.47 | Human | 139.18 | Rhesus | 142.79 | Guinea pig | 196.73 |
| | Horse | 155.37 | Rhesus | 139.35 | Cat | 142.95 | Bovine | 196.71 |
| | Cat | 155.03 | Goat | 139.68 | Dog | 144.24 | Baboon | 196.59 |
| | Bovine | 154.94 | Sheep | 139.81 | Horse | 144.25 | Horse | 196.51 |
| | Baboon | 154.81 | Horse | 139.98 | Rabbit | 144.64 | Cat | 196.14 |
| | Dog | 154.57 | Baboon | 140.07 | Goat | 144.66 | Dog | 196.08 |
| | Goat | 154.51 | Bovine | 140.08 | Baboon | 144.73 | Goat | 196.04 |
| | Sheep | 154.28 | Rabbit | 140.71 | Sheep | 144.75 | Rat | 195.78 |
| | Swine | 154.10 | Dog | 140.81 | Rat | 145.08 | Sheep | 195.40 |
| | Rat | 153.91 | Rat | 140.90 | Bovine | 145.35 | Rabbit | 195.19 |
| | Rabbit | 153.81 | Swine | 141.13 | Swine | 145.46 | Swine | 195.08 |
| | Llama | 152.07 | Camel | 142.02 | Camel | 146.93 | Llama | 193.40 |
| | Camel | 151.90 | Llama | 142.33 | Llama | 147.33 | Camel | 193.04 |

*(Leftmost column: vertical axis labeled "Hydrophobicity" at top with upward arrow, and "Hydrophilicity" at bottom.)*

taker, 1990) or to increased hydrophilicity (Hopp and Woods, 1981; Guy, 1985), depending on the scoring method. However, we ranked combined scores so that the more hydrophobic hemoglobins can be found at the top of the table, whereas the more hydrophilic ones are at the bottom of the table.

Differences in amino acid compositions of hemoglobins of camel and camelids, when compared to other species, were in general similarly interpreted by all four methods used. Camel and llama hemoglobins appear to be the most hydrophilic ones among the hemoglobins of various species investigated.

We used the method of Cowan and Whittaker to generate comparative sequential amino acid hydrophobicity scales (Fig. 2). The arbitrary number assigned to the hydrophobicity of every camel HBA or HBB amino acid residue has been subtracted from the corresponding number of human HBA or HBB molecules; the results are displayed.

Therefore a data point in the >0 region indicates a more hydrophilic, and a point in the <0 region indicates a more hydrophobic residue of the camel HBA or HBB molecule, as opposed to the corresponding residue of human HBA or HBB molecule. Our results indicate that the majority of changes seen increase the hydrophilicity of camel hemoglobin molecules over the human hemoglobin molecules. These changes are fairly evenly distributed within different variable regions of HBA and HBB chains.

## Osmotic behavior of water in human, camel, bovine, and swine erythrocytes

Erythrocytes, like most other mammalian cells, shrink and swell in hyper- or hypoosmolar environments, respectively. The volume changes seen correspond to net water movement across the plasma membrane. We established that erythrocytes are in an osmotic equilibrium with their environment when incubated in mannit solutions of various concentrations at 4°C for 3 h.

We exposed human, camel, bovine, and swine erythrocytes to 150–500 mOsm mannit solutions at 4°C for 3 h. After the incubation, their water contents were determined as described in Materials and Methods. We expressed the erythrocyte water contents as g water/g dry mass and plotted these data against the inverse of media osmolarity (Fig. 3).

According to the van't Hoff equation $\pi V = nRT$, where $\pi$ is the osmotic pressure, $V$ is the volume of water associated with $n$ moles of solute in the erythrocyte, $R$ is the gas constant, and $T$ is the absolute temperature. Considering that all of the water behaves ideally and participates in the osmotic equilibrium, $1/\pi$ must extrapolate to zero. The extent of deviation from zero ($y$-intercept of the curve) indicates that some of the erythrocyte water has reduced osmotic activity over the range of osmotic pressures studied.

The range of osmotic pressure applied was dictated by the osmotic susceptibility of the various erythrocytes. Hemolysis was less than 2% at the end of the experiment at each data point.

Among the erythrocytes of the four species tested, the erythrocytes of camel show the highest osmotically unresponsive water fraction (0.679 g water/g dry mass), followed by swine, bovine, and human. This appears to correspond to the relative hydrophilicites of these hemoglobin molecules (Table 2).

## Erythrocyte water content versus osmotically unresponsive water fraction

Although there is an abundance of data on the erythrocyte water contents, especially for human, for a better comparison we used our own data.

Total erythrocyte water contents were plotted against hemoglobin hydrophobicities (Fig. 4). It is apparent that erythrocytes containing more hydrophilic hemoglobin have lower water contents when compared to erythrocytes with more hydrophobic hemoglobins ($R = -0.83$, SE = 0.17).
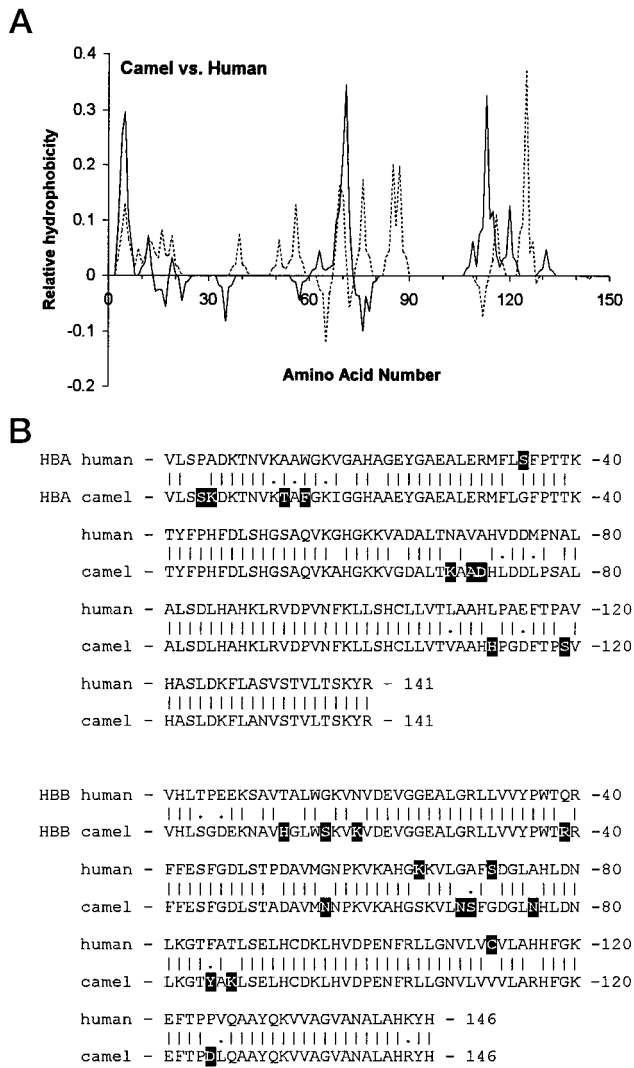
## A



## B

```
HBA human - VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTK -40
            ||  |||||.|.||.| ||  |||||||||||  |||||
HBA camel - VLSSKDKTNVKTAFGKIGGHAAEYGAEALERMFLGFPTTK -40

    human - TYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNAL -80
            ||||||||||||||| ||||| |||| |.||.| ||
    camel - TYFPHFDLSHGSAQVKAHGKKVGDALTKAADHLDDLPSAL -80

    human - ALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAV -120
            |||||||||||||||||||||||||||.||| ||.||| |
    camel - ALSDLHAHKLRVDPVNFKLLSHCLLVTVAAHHPGDFTPSV -120

    human - HASLDKFLASVSTVLTSKYR - 141
            ||||||||| ||||||||||
    camel - HASLDKFLANVSTVLTSKYR - 141


HBB human - VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQR -40
            |||. .|| ||  || || |||||||||||||||||||| |
HBB camel - VHLSGDEKNAVHGLWSKVKVDEVGGEALGRLLVVYPWTRR -40

    human - FFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDN -80
            |||||||||| |||| |||||||| ||| |||.||||||
    camel - FFESFGDLSTADAVMNNPKVKAHGSKVLNSFGDGLNHLDN -80

    human - LKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGK -120
            ||||.| ||||||||||||||||||||||||| ||| ||||
    camel - LKGTYAKLSELHCDKLHVDPENFRLLGNVLVVVLARHFGK -120

    human - EFTPPVQAAYQKVVAGVANALAHKYH - 146
            ||||. .|||||||||||||||||.||
    camel - EFTPDLQAAYQKVVAGVANALAHRYH - 146
```

FIGURE 2    (*A*) Relative hydrophilicities of aligned HBA (——) and HBB (·····) sequences of camel and human. Amino acid positions are displayed on the *x* axis. The hydrophobicities are calculated according to the method of Cowan and Whittaker (1990). The relative hydrophilicities of the compared sequences are displayed on the *y* axis. Positive numbers indicate more hydrophilic amino acid residues in the camel HBA or HBB chain, respectively, whereas negative numbers label more hydrophilic residues in the compared (human) sequences. (*B*) Sequence alignment of human and camel HBA and HBB chains. Vertical lines (|) label identical residues; dots (.) label similar residues. Hydrophilic amino acid substitutions are printed in black background.

Linear regression analysis of osmotically unresponsive water (Fig. 3) versus erythrocyte water (Table 3) revealed a significant ($p < 0.05$) negative relationship ($R = -0.95$). Note that camel had the highest osmotically unresponsive water fraction and the lowest erythrocyte water content.

### Water, potassium, and sodium concentrations of erythrocytes of several mammalian species

Erythrocyte water contents vary between 1.16 and 2.37 g water/g dry mass in the various mammalian species inves-tigated in the present study (Table 3). Most erythrocytes contain ~1.7–2.3 g water/g dry mass, whereas the erythro-cytes of camel contain significantly less (1.16 g water/g dry mass) water. According to the measurements of Weiser and co-workers, the erythrocytes of healthy llamas contain sim-ilarly low quantities of water (1.15–1.31 g water/g dry mass) compared to the camels (Weiser et al., 1992).

There are significant variations in the erythrocyte $K^+$ and $Na^+$ levels among the various species. The sum of the potassium and sodium concentrations also changes remark-ably (Table 3). The erythrocytes of camel contain the lowest concentration of monovalent cations, whereas the "low po-tassium type" (LK type) erythrocytes of dog, cat, and sheep contain the highest total monovalent cation concentration.

We note that the plasma monovalent cation concentra-tions are similar among the species studied ($K^+ = 3.2$–6.2 mM, $Na^+ = 132$–153 mM).

## DISCUSSION

Because of its enormous physiological and pathophysiolog-ical importance, hemoglobin structure and structure changes are studied extensively. Since the discovery of the Glu-Val change in the sixth position of the HBB chain of human hemoglobin (Ingram, 1957), we know that the mutation of a single amino acid may result in dramatic alteration in he-moglobin structure and erythrocyte function/morphology. Thus the remarkable evolutionary conservation of hemoglo-bin amino acid sequence among a number of mammalian species is aimed at the preservation of the key structural features of hemoglobin molecules (Fig. 1). This is needed to retain efficient oxygen binding/release and functionally in-tact erythrocytes.

Although the alterable amino acid residues of hemoglo-bin molecules are restricted to ~13–17 per HBA or HBB chain among the 16 species investigated here, we found significant amino acid composition differences among the hemoglobins of various species (Table 1). Notably, camel and llama hemoglobins contain a higher number of charged amino acid residues than hemoglobins of other species.

Directly related to these changes are the increased hydro-philicities of camel and llama hemoglobins over the hemo-globins of other species (Table 2 and Fig. 2).

Earlier, measurements of osmotically unresponsive water fractions in pig lens fiber cells (Cameron et al., 1988), *Xenopus* oocytes (Cameron et al., 1990), sea urchin egg (Merta et al., 1986), and frog sartorius muscle (Ling and Negendank, 1970) indicated that this osmotically unrespon-sive water fraction may be as low as 31% (frog sartorius muscle) or as high as 92% (lens fiber cells) of the cellular water content.

A comparison of the human, camel, bovine, and swine erythrocytes suggests that the highest osmotically unrespon-sive (bound) water fraction is present in the camel erythro-cytes, followed by swine, bovine, and human erythrocytes (Fig. 3). This—as predicted—agrees with the overall hy-
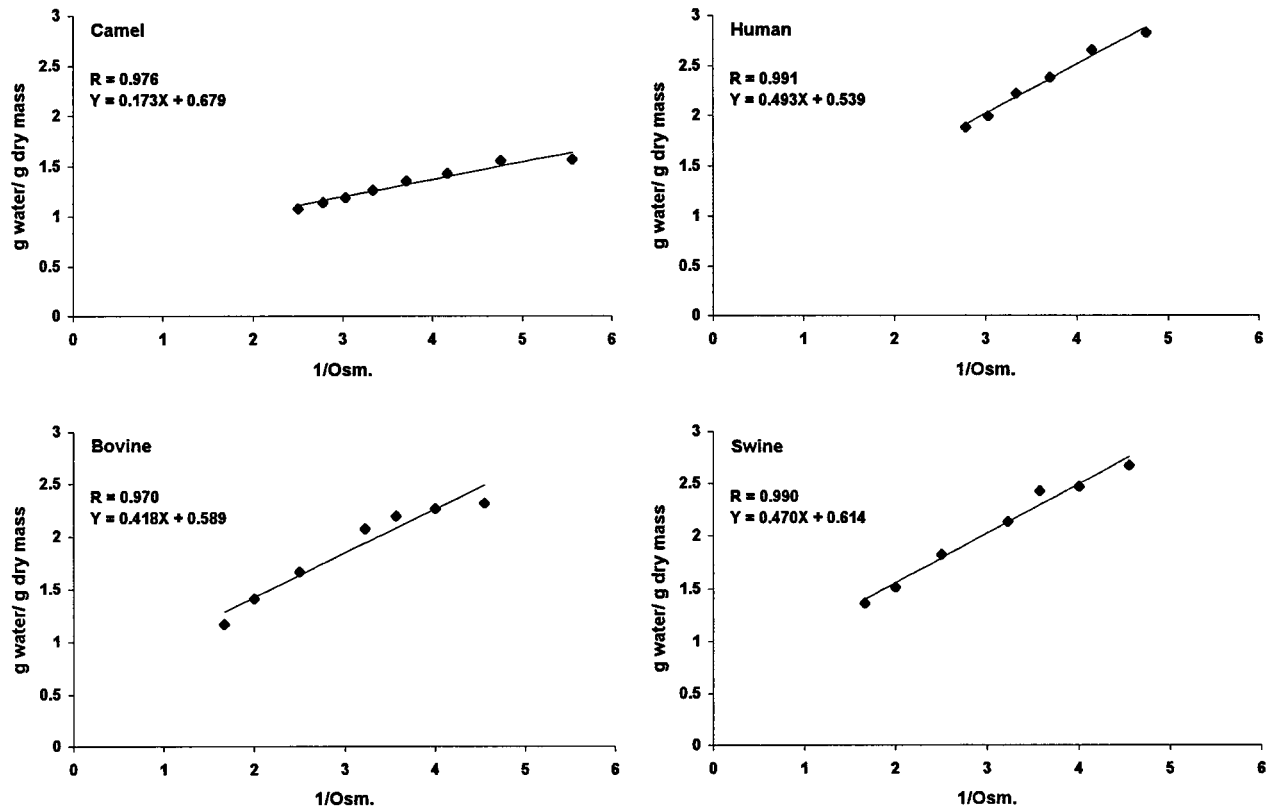
FIGURE 3   The osmotic behavior of camel, human, bovine, and swine erythrocytes. Erythrocytes were incubated in mannit solutions of various molarities at 4°C for 3 h, and their water content was measured as described in Materials and Methods. The calculated $y$ axis intercept indicates osmotically nonresponsive water fractions. Camel: 0.679 g water/g dry mass, or 58.5% of total erythrocyte water; human: 0.539 g water/g dry mass, or 25.8% of total erythrocyte water; bovine: 0.589 g water/g dry mass, or 31.7% of total erythrocyte water; swine: 0.614 g water/g dry mass, or 33.2% of total erythrocyte water.

drophilicity-hydrophobicity rankings of hemoglobins of the species investigated (Table 2). The high osmotically unresponsive water fraction in camel erythrocytes is particularly remarkable, if the low total erythrocyte water content of the camel erythrocyte is considered. More than 58% of the total cellular water is osmotically inactive in the camel erythrocytes. This value is 26% in human, 32% in bovine, and 33% in swine erythrocytes.

Next we asked if the erythrocyte water content relates directly to increased hemoglobin hydrophilicity. In agreement with earlier observations, we found that erythrocyte water contents of camels and camelids are very low (1.1–1.3 g water/g dry mass) (Weiser et al., 1992; Perk, 1963, Yagil,
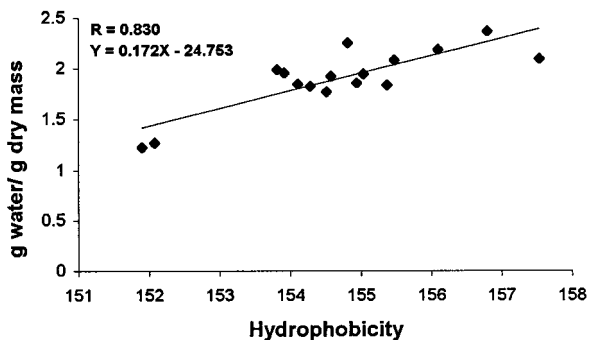


FIGURE 4   The relationship between hemoglobin hydrophobicity (hydrophilicity) and erythrocyte water levels. The hydrophobicity score of various hemoglobins is shown on the $x$ axis (Cowan and Whittaker, 1990). The actual erythrocyte water contents are displayed on the $y$ axis.

**TABLE 3   $K^+$, $Na^+$, and water contents of various erythrocytes**

| Species | $n$ | $K^+ + Na^+$ (mM) | $H_2O$ (g water/g dry mass) |
|---|---|---|---|
| *Camel* | 3 | *128.4 ± 2.5* | *1.16 ± 0.05* |
| Human | 5 | 164.8 ± 3.4 | 2.09 ± 0.05 |
| Bovine | 5 | 153.8 ± 4.0 | 1.86 ± 0.07 |
| Swine | 6 | 192.1 ± 2.2 | 1.85 ± 0.09 |
| Rabbit | 6 | 177.1 ± 1.9 | 1.99 ± 0.05 |
| Rhesus | 1 | 184.8 | 2.37 |
| Baboon | 4 | 161.6 ± 12.1 | 2.25 ± 0.11 |
| Goat | 6 | 152.0 ± 12.0 | 1.81 ± 0.14 |
| Sheep, LK | 16 | 193.5 ± 4.6 | 1.85 ± 0.07 |
| Horse | 6 | 171.5 ± 1.2 | 1.83 ± 0.05 |
| Dog | 6 | 191.2 ± 3.5 | 1.93 ± 0.03 |
| Cat | 3 | 198.3 ± 5.3 | 1.95 ± 0.04 |
| Rat | 3 | 166.3 ± 6.0 | 1.96 ± 0.06 |
| Hamster | 3 | 166.0 ± 15.1 | 2.09 ± 0.06 |
| Guinea pig | 3 | 165.3 ± 2.6 | 2.19 ± 0.01 |

1974; Ellory and Tucker, 1983) (Table 3). The difference in water contents of erythrocytes among other mammals is less striking; water contents differ between 1.7 and 2.4 g water/g dry mass.

We plotted the erythrocyte water contents of the investigated species against the hydrophilicities of their hemoglobins according to the ranking of Cowan and Whittaker (1990) (Fig. 4). Regression analysis resulted in an inverse linear relationship between hemoglobin hydrophilicity and erythrocyte water content ($R = -0.83$, SE = 0.17).

Erythrocyte $K^+$ and $Na^+$ levels of various species were also measured and compared. We found that camel erythrocytes contain the lowest monovalent cation concentration ($K^+ + Na^+$) among the mammals investigated (Table 3).

Because the $K^+$ and $Na^+$ composition of the camel plasma is similar to those of other mammals, this interesting phenomenon may also relate to the special properties of camel hemoglobins. Namely, the high basic amino acid content of the camel hemoglobin and the very high erythrocyte hemoglobin concentration result in a high net positive fixed charge density, which repels $K^+$ and $Na^+$ in a Donnan equilibrium-like manner. In addition, more pronounced water structuring around the camel hemoglobin may affect the intra/extracellular partition of hydrated ions like $K^+$ and $Na^+$.

The characteristic differences seen in the variable parts of hemoglobin sequences of camel and camelids are likely to be the results of evolutionary adaptation mechanisms. Increased number of charged amino acid residues results in increased hemoglobin hydrophilicity and greater resistance to osmotic dehydration or hyperhydration.

Consequently, in addition to physiological regulatory mechanisms, which help the survival of desert animals, there may be a molecular-cellular level of defense against dehydration.

## REFERENCES

Bull, H., and K. Breeze. 1974. Surface tension of amino acid solutions: a hydrophobicity scale of the amino acid residues. *Arch. Biochem. Biophys.* 161:665–670.

Cameron, I. L., E. Contreras, G. D. Fullerton, M. Kellermayer, A. Ludany, and A. Miseta. 1988. Extent and properties of nonbulk "bound" water in crystalline lens cells. *J. Cell. Physiol.* 137:125–132.

Cameron, I. L., K. M. Kanal, C. R. Keener, and G. D. Fullerton. 1997. A mechanistic view of the non-ideal osmotic and motional behavior of intracellular water. *Cell Biol. Int.* 21:99–113.

Cameron, I. L., P. Merta, and G. D. Fullerton. 1990. Osmotic and motional properties of intracellular water as influenced by osmotic swelling and shrinkage in *Xenopus* oocytes. *J. Cell. Physiol.* 142:592–602.

Clegg, J. S. 1984. Properties and metabolism of the aqueous cytoplasm and its boundaries. *Am. J. Physiol.* 246:R133–R151.

Cowan, R., and R. G. Whittaker. 1990. Hydrophobicity indices for amino acid residues as determined by high-performance liquid chromatography. *Pept. Res.* 3:75–80.

Ellory, J. C., and E. M. Tucker. 1983. Cation transport in red blood cells. *In* Red Blood Cells of Domestic Mammals. Elsevier, Amsterdam. 291–314.

Feng, D. F., M. S. Johnson, and R. F. Doolittle. 1985. Aligning amino acid sequences: comparison of commonly used methods. *J. Mol. Evol.* 21:112–125.

Guy, H. R. 1985. Amino acid side-chain partition energies and distribution of residues in soluble proteins. *Biophys. J.* 47:61–70.

Higgins, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene.* 73:237–244.

Higgins, D. G., and P. M. Sharp. 1989. Fast and sensitive multiple sequence alignment on a microcomputer. *CABIOS.* 5:151–153.

Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* 78:3824–3828.

Ingram, V. M. 1957. Gene mutations in hemoglobin: the chemical difference between normal and sickle cell hemoglobin. *Nature.* 180:326–328.

Janin, J. 1979. Surface and inside volumes in globular proteins. *Nature.* 277:491–492.

Keener, C. R., G. D. Fullerton, I. L. Cameron, and J. Xiong. 1995. Solution nonideality related to solute molecular characteristics of amino acids. *Biophys. J.* 68:291–302.

Ling, G. N., and W. Negendank. 1970. The physical state of water in frog muscles. *Phys. Chem. Phys.* 2:15–23.

Merta, P. J., G. D. Fullerton, and I. L. Cameron. 1986. Characterization of water in unfertilized and fertilized sea urchin eggs. *J. Cell. Physiol.* 127:439–447.

Myers, E. W., and W. Miller. 1988. Optimal alignments in linear space. *CABIOS.* 4:11–17.

Perk, K. 1963. The camel's erythrocyte. *Nature.* 200:272–273.

Radzicka, A., and R. Wolfenden. 1988. Comparing the polarities of amino acids: side chain distribution coefficients between the vapor phase, cyclohexane, 1-octanol, and neutral aqueous solution. *Biochemistry.* 27:1664–1670.

Weiser, M. G., M. J. Fettman, D. Van Houten, L. Johnson, and F. Garry. 1992. Characterization of erythrocytic indices and serum iron values in healthy llamas. *Am. J. Vet. Res.* 53:1776–1779.

Wheatley, D. N. 1991. Water: biology's forgotten molecule. *Biologist.* 38:45–49.

Yagil, R., U. A. Sod-Moriah, and N. Meyerstein. 1974. Dehydration and camel blood. II. Shape, size, and concentration of red blood cells. *Am. J. Physiol.* 226:301–304.

Zimmerman, S., A. Zimmerman, G. D. Fullerton, R. Luduena, and I. L. Cameron. 1993. Solute/solvent interaction corrections account for nonideal freezing point depression. *J. Biochem. Biophys. Methods.* 26:61–70.