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Osmotic Effects of Protein Polymerization: Analysis of Volume Changes in Sickle Cell Anemia Red Cells following Deoxy-Hemoglobin S Polymerization

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Summary. Polymerization-depolymerization of proteins within cells and subcellular organelles may have powerful osmotic effects. As a model to study these we analyzed the predicted volume changes following hemoglobin (Hb) S polymerization in sickle cell anemia (SS) red cells with different initial volumes. The theoretical analysis predicted that dehydrated SS red cells may sustain large polymerization-induced volume shifts whose direction would depend on whether or not small solutes were excluded from polymer-associated water. Experiments with SS cells from promptly fractionated venous blood showed oxygenation-induced swelling, maximal in the densest cells, in support of nonexclusion models. The predicted extent of cell dehydration on polymerization was strongly influenced by factors such as the dilution of residual soluble Hb and the increased osmotic contribution of Hb in cells dehydrated by salt loss, largely overlooked in the past. The osmotic effects of polymer formation may thus play an important part in microcirculatory infarction by dense SS cells, as they become even denser and stiffer during deoxygenation in the capillaries.

Key Words protein polymerization · cell volume and osmolality regulation · polymer water · sickle cells · hemoglobin S polymerization · red cells

Introduction

Cells and intracellular organelles enclose solutions which contain proteins, often in very high concentrations, which contribute significantly to the osmolality of these compartments. For hemoglobins of diverse origins, this contribution has been shown to increase with the second or third power of the protein concentration [1–3, 11–13, 17–18, 21, 24, 28, 36–38]. Changes in protein concentration within intracellular domains may arise from changes in cell or organelle volume or by polymerization-depolymerization of major protein components. The resulting changes in osmolality and their possible effects may not have been fully appreciated. We therefore analyzed in detail the osmotic effects accompanying reversible polymerization of Hb S in sickle cell anemia red cells (SS cells), for which much of the required physico-chemical information is available [9, 13].

Polymerization of deoxygenated Hb S reduces the cell concentration of osmotically active particles, and was therefore assumed to induce cell shrinkage [32]. The estimated volume shifts were considered "small but potentially detectable" [15]. Previous results in the literature, however, are confusing and conflictive; they variably report shrinkage [27], swelling [23] or no volume change [16] of SS cells on deoxygenation. Some possible sources of these discrepancies were discussed by Fabry and Nagel [15]; additional factors include the difficulties of measuring packed volumes or densities of rigid and deformed cells. Thus, the direction and extent of cell volume changes following Hb S polymerization and depolymerization remained an open issue.

Since the generally accepted current estimate of the concentration of Hb within the polymer, C_p (69 ± 6 g/dl), is considerably lower than that of Hb in its exclusion volume (34 g/0.25 dl ≈ 136 g/dl), polymers of deoxy-Hb S must retain a substantial proportion of water within their lattice structure [9, 13, 32, 40]. Experimental results suggest that this polymer-associated water is inaccessible to soluble Hb and presumably to other macromolecules [9, 13, 30, 39, 40]. But the extent to which small solutes may be globally or selectively excluded from polymer water was unknown, and the information is necessary for the analysis of the osmotic effects of Hb S polymerization. As shown below, the issue becomes particularly important when considering dehydrated SS cells in which polymer water may comprise a large fraction of the cell water. The inclusion or displacement of small osmoticants in the polymer water compartment could thus have major effects on the cell volume changes which accompany polymerization and depolymerization.

In this paper we perform a theoretical analysis of the possible osmotic effects of deoxy-Hb S polymerization. The analysis predicts that SS cells would swell or shrink on polymerization-depolymerization depending on the access small solutes have to polymer-associated water. To resolve whether cells swell or shrink on depolymerization we measured oxygenation-induced density changes of SS cells from freshly drawn venous blood.

Materials and Methods

The analysis of the osmotic effect of hemoglobin S polymerization centers on the change in SS cell volume when a fraction P of its Hb polymerizes and, therefore, ceases to contribute osmotic particles. We seek to derive expressions to predict the changes in cell volume, osmotic coefficient and ideal osmolality of residual unpolymerized Hb, and fraction of cell osmoticants within polymer water, as functions of polymer fraction and prepolymerization cell volumes. To predict differences in outcome which depend on the extent to which the cell's low molecular weight (MW) osmoticants equilibrate within polymer water, the expressions incorporate an inclusion factor as a variable to be tested (*see h* in Glossary). Before deriving the equations we present a glossary of symbols, and a list of definitions and assumptions listed as *Dn* (n = 1 to 7). The nomenclature follows and extends that used in previous models of cell volume and ion content regulation in epithelia [25], red cells [24] and reticulocytes [26].

Glossary

1, 2: Subscripts indicating values of variables before and after Hb S polymerization, respectively.

b, c: Virial coefficients of linear and quadratic terms, respectively, in empirical equation for the osmotic coefficient of Hb (f). Values used for b and c: 0.0645 and 0.0258, respectively [17].

f: Osmotic coefficient of Hb (imosmol/mmol; *see* D1 below for definition of imosmole).

h: Inclusion factor; defines full or partial exclusion ($h = 0$ or $0 < h < 1$, respectively) of all low MW cell osmoticants from polymer water, or full osmotic equilibration ($h = 1$) between cytosolic and polymer water.

f_Σ: Fraction of total low MW cell osmoticants that would be contained in polymer water at equilibrium if there is no exclusion ($h = 1$).

C_P: Concentration of deoxy-Hb S within the polymer (g/dl or mM), assumed constant for the purposes of this analysis. Currently accepted value: 69 g/dl, equivalent to 10.7 mM Hb tetramer, MW 64,500 [39].

C_H: Concentration of Hb in its own molar volume, i.e., the inverse of the specific volume of Hb (g/dl or mM). Value used; 136 g/dl, equivalent to 21.1 mM [10].

C_{iso}: Ideal osmolality of extracellular and cytosolic fluids, assumed constant (imosmolal). Value used: 295 imosmolal.

C_s: Concentration of soluble oxy and deoxy-Hb in the cytosol, excluding polymer volume (g/dl or mM).

C_{sat}: Concentration of soluble deoxy-Hb S at equilibrium with polymer (g/dl or mM). This represents the solubility of deoxy-Hb S (*see* Eaton & Hofrichter [13], and references therein). It varies with temperature but is assumed here to be independent of the total Hb concentration; value used for 37°C: 17 g/dl, equivalent to 2.6 mM.

Q_{Hb}: Constant representing the average amount of Hb in one liter of normal-volume, fully packed red cells (g or mmol). Q_{Hb} is assumed to be independent of SS cell volume and to remain invariant during polymerization-induced volume changes in SS cells. Value used: 340 g (or g/loc), equivalent to 5.27 mmol (or mmol/loc).

ΣQ: Total amount of low MW cell osmoticants (imosmol/loc or imosmol/340 g Hb). Unlike Q_{Hb}, ΣQ varies with the original SS cell volume, but is assumed to remain constant during polymerization-induced volume changes (*see D2* below).

V: Total volume of cell water (liter/loc or liter/340 g Hb).

V^T: Total cell volume (liter/loc or liter/340 g Hb).

V_H: Total volume occupied by Hb molecules within the cell (liter/loc or liter/340 g Hb). V_H was shown to remain constant during polymerization [22]. Value used: 0.25 liter/loc or 0.25 liter/340 g Hb.

V^P: Total volume of polymer (liter/loc or liter/340 g Hb).

V_H^P: Volume occupied by polymerized Hb (liter/loc or liter/340 g Hb).

V_w^P: Volume occupied by polymer-associated water (liter/loc or liter/340 g Hb).

P: Fraction of polymerized Hb ($0 \le P \le 1$).

P_{max}: Maximal fraction of polymerized Hb which can be formed within a fully deoxygenated SS cell. The limitation $P_{max} < 1$ arises because the concentration of unpolymerized Hb in the cytosol cannot fall below the solubility of deoxy-Hb S (C_{sat}).

MCHC or MC: Mean cell Hb concentration (g/dl); the abbreviated form MC is used as column heading in Table 1. *See also D7*.

δ: Cell density (g/liter).

$\Delta V/V$: Polymerization-induced change in cell volume relative to the initial total volume of the cell (±%).

O: Ideal osmolality of Hb (imosmolal).

Definitions and Main Assumptions

D1. The molal concentration of a solute multiplied by its osmotic activity coefficient is described as its "ideal osmolality" or "iosmolality." By definition then, this concentration will obey $\pi = qRT$ [iosmolality], where R and T are the gas constant and absolute temperature, respectively, π is the partial osmotic pressure exerted by the solute, and q is a units-conversion factor. For Hb, for instance, the iosmolality is given by $f \cdot Q_{Hb}/V$; for the low MW cell osmoticants, ΣQ, by $\Sigma Q/V$, both in units of "imosmolal." Osmotic equilibria can then be expressed as equalities between iosmolal concentrations.

D2. The main low MW solutes (ΣQ) in physiological conditions are K^+, Cl^-, HCO_3^-, Na^+, Mg^{2+}, 2,3-DPG, nucleotides and glycolytic metabolites. We consider here whether ΣQ may be assumed to be constant during polymerization-depolymerization. The amounts and osmotic activity coefficients of ΣQ components may vary due to concentration changes, to binding-dissociation during oxy-deoxy Hb transitions, to metabolism, or to transport. Concentration-dependent changes in the osmotic activity coefficients of ΣQ components within the range of the cell volume shifts considered here are orders of magnitude below those of the cell proteins, largely Hb. Binding-dissociation shuttles 2,3-DPG and ATP between Mg^{2+} and deoxy-Hb with relatively minor changes in overall particle balance. Hours, rather than minutes or seconds are required to expose any global ion content or metabolic changes in SS discocytes and denser cell fractions treated with

continuous or alternating full deoxygenation pulses in plasma or plasma-like media [5–7, 41–42]. We may therefore assume that over the seconds-minutes time scale of the polymerization-depolymerization-induced volume shifts analyzed here, the changes in the global osmotic activity of the low MW cell solutes, represented by ΣQ, are minor and can be neglected.

D3. Dehydrated SS cells have a reduced salt content. By whatever mechanism, oxygenated SS cells with a wide distribution of increased density must have sustained a net salt loss. Thus, although ΣQ may be assumed to remain constant during polymerization (D2), it must vary in SS cells in proportion to their prepolymerization volumes (V_1).

D4. When a cell contains polymer, its cytoplasm is assumed to be composed of two distinct phases, cytosol and polymer, whose volumes add up to the total cell volume. Whatever the extent of osmotic equilibration between polymer and cytosol, represented by the value of h, water transport is assumed to be sufficiently fast to secure unmeasurably rapid equilibration between all intracellular and extracellular compartments. Polymerization-depolymerization-induced cell volume shifts may thus be derived from equations which assume instant osmotic equilibria.

D5. Soluble Hb is excluded from polymer water [9, 30, 35, 36, 39, 40].

D6. Within the experimental errors of most mean cell density measurements, the densities of water and of protein-free salt solutions in the isotonic range may be assumed to be indistinguishable from 1 kg/liter; therefore, 1 liter of solution ≈ 1 kg of solvent. To accommodate 340 g of Hb in 0.25 liter [10], the mean density of normal red cells was set at 1090 g/loc. The volume of cell water per liter normal cells (loc) is therefore (1.090–0.340) = 0.750 liter/loc, neglecting contributions to volume and weight from membrane, cytoskeleton and salts.

D7. MCHC is used to represent both the mean cell Hb concentration of a real density fraction of red cells and the Hb concentration in a single model red cell as used in the theoretical analysis. In the latter case, "mean" has no real meaning, but the term MCHC is retained to emphasize the relation between theory and experimental test or application.

Derivation of the Equations

Before polymerization, the volume of cell water, (V_1, in liter/loc or in liter/340 g Hb), is given by (*D1–D4*)

$$V_1 = \frac{\Sigma Q + f_1 Q_{Hb}}{C_{iso}} \tag{1}$$

where f_1, the osmotic coefficient of hemoglobin in units of iosmol/mol, is described by the empirical equation [17, 24]

$$f_1 = 1 + b\left(\frac{Q_{Hb}}{V_1}\right) + C\left(\frac{Q_{Hb}}{V_1}\right)^2. \tag{2}$$

ΣQ and C_{iso} are assumed invariant during polymerization (*D2*); cells with different initial V_1 will have different ΣQ (*D3*), whereas C_{iso} is constant throughout. From Eq. (1) we compute ΣQ for each V_1

$$\Sigma Q = V_1 C_{iso} - f_1 Q_{Hb}. \tag{3}$$

Before polymerization, the total cell volume, V_1^T, consists of one soluble phase in which the Hb molecules occupy about 0.25 liter per liter of normal volume cells. After polymerization of a fraction P of the Hb, the total cell volume (V_2^T) becomes partitioned in two phases, polymer and cytosol, which include four distinct compartments: the volumes occupied by Hb (V_H^P) and water or protein-free solution (V_w^P) within the polymer, and those occupied by residual soluble Hb and solution in the cytosolic phase. The volume occupied by polymer can be estimated from

$$V^P = \frac{PQ_{Hb}}{C_P} \tag{4}$$

where $C_P \approx 69$ g/dl (69 ± 6 g/dl [13, 35, 39]), equivalent to about 11 mM, is the reported concentration of Hb within the polymer, as measured in solubility experiments by infrared spectroscopy on the turbid pellets [39]. V^P comprises the volume of Hb in the polymer, V_H^P, and the volume of water (or solution) within the polymer, V_w^P.

Since the total volume of a solution of Hb S was shown to be unchanged by polymerization [22], the total volume occupied by soluble and polymerized Hb, V_H, must remain constant at about 0.25 liter/340 g Hb. V_H^P can therefore be calculated from

$$V_H^P = \frac{PQ_{Hb}}{C_H} \tag{5}$$

where C_H, the inverse of the specific volume of Hb, represents the concentration of Hb in its own exclusion volume. V_w^P can be estimated from

$$V_w^P = V^P - V_H^P \tag{6}$$

and is given by

$$V_w^P = PQ_{Hb}\left(\frac{1}{C_P} - \frac{1}{C_H}\right). \tag{7}$$

Soluble Hb is presumably excluded from polymer water (*D5*), but the extent to which low MW solutes (ΣQ) are excluded from it is unknown. To analyze predictions for any degree of exclusion of ΣQ components from V_w^P, the simplest strategy is first to compute the maximal fraction of ΣQ that could be contained within V_w^P, f_Σ, for each value of P, at osmotic equilibrium. We can then multiply ($f_\Sigma \cdot \Sigma Q$) by an inclusion factor h, between 0 (total exclusion of ΣQ from V_w^P) and 1 (total equilibration). The condition of osmotic equilibrium (*D4*) between cytosolic and polymer water compartments is described by

$$\frac{f_\Sigma \Sigma Q}{V_w^P} = C_{iso}. \tag{8}$$

From Eqs. (7) and (8) we compute f_Σ

$$f_\Sigma = \frac{PQ_{Hb}C_{iso}}{\Sigma Q}\left(\frac{1}{C_P} - \frac{1}{C_H}\right). \tag{9}$$

With f_Σ, we can now formulate osmotic equilibrium (*D4*) between the cytosol (V_2) and extracellular medium for the general case using the inculsion factor h

$$\frac{(1 - hf_2)\Sigma Q + f_2(1 - P)Q_{Hb}}{V_2} = C_{iso} \quad (10)$$

where f_2 is

$$f_2 = 1 + b\left(\frac{(1 - P)Q_{Hb}}{V_2}\right) + C\left(\frac{(1 - P)Q_{Hb}}{V_2}\right)^2. \quad (11)$$

Defining

$$w = \frac{(1 - P)Q_{Hb}}{V_2} \quad (12)$$

Eqs. (10)–(12) combine to give

$$C_{iso} - \frac{(1 - hf_2)\Sigma Q}{V_2} - w(1 + bw + cw^2) = 0. \quad (13)$$

For any given P and h, we can now compute V_2, the only remaining unknown in Eq. (13). With V_2, we can calculate the total cell volume after polymerization, V_2^T, from

$$V_2^T = V_H + V_w^P + V_2. \quad (14)$$

To analyze predictions derived from this analysis and design experimental tests, we need to output results to tables and graphs containing, besides the variables defined so far, easily measurable quantities such as mean corpuscular hemoglobin concentration (MCHC, *D*6, *D*7) and cell density (δ, *D*6). δ and MCHC are linked to cell water volume (V) by

$$\delta = \frac{0.340 + V}{0.25 + V} \quad (15)$$

$$\text{MCHC} = \frac{34}{0.25 + V}. \quad (16)$$

Application of the Equations

The total cell volume before polymerization (V_1^T, in liter/loc) is thus given by $V_1^T = 0.25 + V_1$; after polymerization, $V_2^T = 0.25 + V_w^P + V_2$.

By systematically varying initial cell volumes (V_1 or V_1^T), and the fraction of polymerized Hb (P) in the above set of equations, we can construct tables of cell volumes and related variables (V, V^T, MCHC, δ), soluble Hb concentration in cytosol (C_s), osmotic coefficients of Hb (f), and Hb isomolality ($f \cdot (1 - P) \cdot Q_{Hb}/V$), before polymerization ($P = 0$, subindex 1) and after ($P > 0$, subindex 2), for any value between 0 and 1 of the inclusion factor h. This method was used to produce the data sets selected for Table 1 and for the figures.

To analyze the relatively slow, secondary shifts in cell pH, volume and ion contents following abrupt polymerization, the effects of Hb polymerization were also simulated using the integrated red cell model of Lew and Bookchin [24] with maximal sickling-induced permeabilization [8]. The results showed that, within the hour following sudden polymerization-depolymerization, the magnitude of the secondary shifts were negligible compared with polymerization-induced changes. Use of a comprehensive model was thus deemed unnecessary for the present analysis, and the assumption in *D*2 was considered justified.

With the analysis of the osmotic effects of deoxy-Hb S polymerization described by Eqs. (1)–(16), the changes in cell volume and related variables can be predicted for any arbitrary values of the polymer fraction P, the initial MCHC and the inclusion factor, h. But not all these values would be meaningful. The fraction of polymerized Hb increases gradually with deoxygenation [29, 31], but there is a limit to the maximal fraction of polymer that can be formed within any fully deoxygenated cell (P_{max}): this limit is determined by the condition that the concentration of unpolymerized Hb at equilibrium with polymer should not fall below the solubility of deoxy-Hb S (C_{sat}). All the results reported are therefore defined for values of P up to P_{max} only, by imposing the condition that the concentration of residual soluble Hb in the cytosol (C_s) should not fall below C_{sat} ($C_{sat} \geq 16$ g/dl at 37°C [13, 33]).

Measurement of the Oxygenation-Induced Changes in the Density Profile of SS Cells from Freshly Drawn Venous Blood

As briefly discussed in the Introduction, the available experimental evidence on the direction of the polymerization-induced volume changes of SS cells is inconclusive. Our analysis (*see* Results) predicts cell swelling or shrinking depending on whether or not low molecular weight cell solutes are excluded from polymer-associated water. Since there is no knowledge yet of the exclusion state of low MW solutes, opposite volume changes remain theoretically possible. It was therefore crucial to resolve the issue experimentally so as to circumscribe the theoretical analysis to conditions that would apply to circulating SS cells. A new experimental approach was clearly required, and the following experiments were designed to provide a qualitative answer.

Samples of SS venous blood were taken directly into partly evacuated tubes containing EGTA (final concentration 4 mM), and the red cell density distribution was measured immediately by layering aliquots on each of three Stractan solutions with densities 1.125, 1.118 and 1.106 in Eppendorf tubes, centrifuging for 5 min at 12000 × g, and measuring the fraction of Hb in the pellet. Part of the original blood sample was equilibrated at 37°C for 25 min with 5.6% CO_2 in oxygen, and the density distribution of the cells was determined again.

Results and Analysis

Predicted Effects of Intracellular HB S Polymerization on Cell Volume

Table 1 and Fig. 1 show the predicted effects of an increasing fraction of polymerized Hb S on the osmotic parameters of SS cells, when non-Hb solutes are either fully equilibrated with polymer water (h = 1), or excluded from it partially (h = 0.6 in Table 1, or 0.5 in Fig. 1) or totally (h = 0). Analysis of the effects on cell volume indicates that if there were full exclusion of all non-Hb solutes from the polymer water compartment, Hb polymerization should cause cell swelling, whereas with full equilibration of low MW cell solutes the cells would shrink

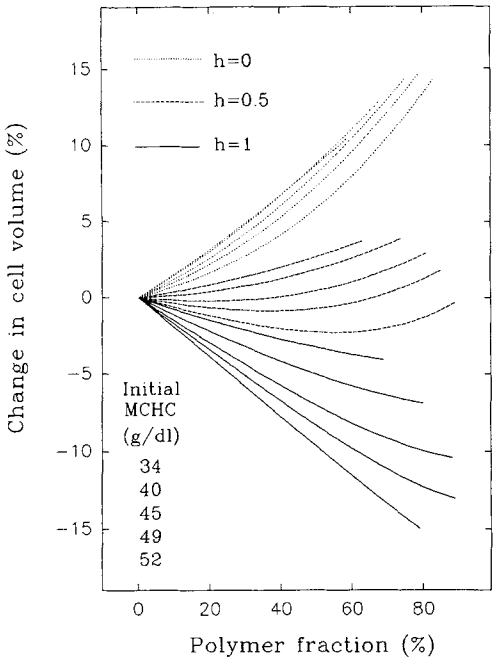

Fig. 1. Predicted changes in SS cell volume induced by Hb polymerization, as a function of polymer fraction. Three models are shown, representing different extents of exclusion of low molecular weight osmoticants from polymer water: $h = 1$ (continuous lines), full inclusion; $h = 0$ (dotted lines), full exclusion; and $h = 0.5$ (dashed lines), partial (50%) exclusion. Within each five-curve set, the curves from top to bottom represent predicted volume changes for cells with initial MCHCs of 34, 40, 45, 49 and 52 g/dl

on polymerization at all initial cell Hb concentrations. With partial solute exclusion, it depends on the initial MCHC whether polymerization results in cell shrinkage or swelling. The lesser the exclusion, the lower the initial MCHC at which polymerization leads to cell shrinkage rather than swelling.

The different lengths of the curves in Fig. 1 and the decreasing range of cell volumes scanned in Table 1 at the higher polymer fractions, both result from the requirement (included in the computations) that at equilibrium, the residual Hb concentration in the cytosol must equal (in fully deoxygenated cells) or exceed (in partially deoxygenated cells) the solubility of deoxy-Hb S ($C_s \geq C_{sat}$). The resulting maximal polymer fraction represents P_{max} for each initial MCHC. The general trend is for P_{max} to increase with initial MCHC at all values of h, since in dehydrated cells, a smaller fraction of total cell Hb is required to have a soluble [Hb] equal to C_{sat}. Such an increase in P_{max} with cell MCHC was previously observed [29, 31]. The exception seen here, a limiting P_{max} in the bottom curve of Fig. 1, will be analyzed with the results in Fig. 3 below.

Effects of Oxygenation of the Density Distribution of SS Red Cells from Freshly Drawn Venous Blood

Analysis of the predictions in Table 1 and Fig. 1 indicate that the direction of SS cell volume changes upon polymerization and depolymerization could help define the real distribution of low molecular weight solutes in polymer water. Our new approach to assess those volume shifts was based on our predictions that at venous oxygen tensions, the relatively denser SS cells may contain sufficient polymer to show detectable swelling or shrinkage after oxygenation. Since rapid measurements of the cells' density before and after oxygenation of fresh venous blood involve fewer and less complex experimental manipulations than those used in previous investigations of polymerization-induced volume shifts [15, 16, 23, 27], the results should provide more reliable clues about the distribution of low MW solutes in polymer water.

The results of six such experiments are shown in Table 2. Despite large differences in the original density distribution of SS red cells from different donors, oxygenation of the fresh venous blood resulted in hydration of cells within each of the relatively high density fractions explored. Within that range, the denser the original fraction, the larger the proportion of cells shifting to lower density. Thus the denser fractions of SS cells are relatively dehydrated at venous oxygen tensions and become rapidly hydrated upon full oxygenation. Since only swelling was detected, over the entire density range investigated, the results suggest that the low MW ions and solutes that comprise most of the cell's osmoticants are not globally excluded from polymer water. Further analysis of the theoretical predictions will therefore be conducted using only the nonexclusion model ($h = 1$), although the present results do not rule out partial or selective exclusion of ΣQ components from polymer water or global exclusion of low MW osmoticants from polymers other than those of Hb S.

Predicted Effects of HB S Polymerization on the Osmotic Parameters of SS Cells (Model with no Exclusion of Low MW Solutes from Polymer Water; $h = 1$)

Table 1 and Fig. 2 report predicted changes in a variety of osmotic parameters of SS cells as a function of polymer fraction. It can be seen that cell shrinkage increases with polymer fraction and initial MCHC, and that the higher the initial MCHC, the steeper the slope of the volume decrease with poly-

Table 1. Osmotic parameters of SS cells as a function of the fraction of polymerized deoxy-hemoglobin S (P), the inclusion factor (h) and the total initial cell volume (V_1^T)

V_1^T	V_2^T	V_1	V_2	ΣQ	$\Delta V/V$	MC_1	MC_2	C_s	f_1	f_2	O_1	O_2	$h \cdot f_\Sigma$
$h = 1$													
$P = 0.20$; $V_w^P = 49$ ml/loc													
650	625	400	326	85	−3.89	52.3	54.4	51.7	6.33	6.14	83.4	79.4	0.169
750	728	500	429	124	−2.97	45.3	46.7	43.2	4.55	4.12	47.9	40.5	0.116
850	831	600	533	158	−2.22	40.0	40.9	37.1	3.56	3.13	31.2	24.8	0.090
950	934	700	635	191	−1.69	35.8	36.4	32.6	2.95	2.56	22.2	17.0	0.075
1050	1036	800	738	223	−1.32	32.4	32.8	29.0	2.54	2.21	16.8	12.6	0.064
1150	1138	900	839	254	−1.06	29.6	29.9	26.2	2.26	1.98	13.2	9.9	0.056
$P = 0.40$; $V_w^P = 97$ mol/loc													
650	600	400	253	85	−7.75	52.3	56.7	50.7	6.33	5.85	83.4	73.2	0.338
750	707	500	360	124	−5.74	45.3	48.1	40.0	4.55	3.56	47.9	31.3	0.232
850	815	600	467	158	−4.18	40.0	41.7	33.0	3.56	2.62	31.2	17.7	0.181
950	920	700	573	191	−3.13	35.8	36.9	28.2	2.95	2.14	22.2	11.8	0.150
1050	1025	800	677	223	−2.43	32.4	33.2	24.7	2.54	1.86	16.8	8.7	0.129
1150	1128	900	781	254	−1.94	29.6	30.2	21.9	2.26	1.68	13.2	6.8	0.113
$P = 0.60$; $V_w^P = 146$ ml/loc													
650	575	400	179	85	−11.54	52.3	59.1	48.7	6.33	5.32	83.4	62.5	0.507
750	689	500	293	124	−8.16	45.3	49.4	34.6	4.55	2.80	47.9	20.1	0.348
850	801	600	405	158	−5.77	40.0	42.4	26.9	3.56	2.03	31.2	10.6	0.271
950	909	700	514	191	−4.27	35.8	37.4	22.2	2.95	1.70	22.2	7.0	0.225
1050	1015	800	620	223	−3.30	32.4	33.5	18.9	2.54	1.52	16.8	5.2	0.193
$P = 0.80$; $V_w^P = 194$ ml/loc													
750	675	500	231	124	−9.96	45.3	50.3	24.2	4.55	1.83	47.9	8.3	0.463
850	792	600	347	158	−6.87	40.0	43.0	17.1	3.56	1.43	31.2	4.3	0.362
V_1^T	V_2^T	V_1	V_2	ΣQ	$\Delta V/V$	MC_1	MC_2	C_s	f_1	f_2	O_1	O_2	$h \cdot f_\Sigma$
$h = 0.60$													
$P = 0.20$; $V_w^P = 49$ ml/loc													
650	639	400	340	85	−1.74	52.3	53.2	50.4	6.33	5.76	83.4	71.4	0.101
750	744	500	446	124	−0.77	45.3	45.7	42.1	4.55	3.92	47.9	37.1	0.070
850	849	600	550	158	−0.13	40.0	40.1	36.3	3.56	3.01	31.2	23.0	0.054
950	952	700	654	191	+0.24	35.8	35.7	31.9	2.95	2.49	22.2	16.0	0.045
1050	1055	800	756	223	+0.46	32.4	32.2	28.4	2.54	2.16	16.8	12.0	0.039
1150	1157	900	858	254	+0.58	29.6	29.4	25.7	2.26	1.94	13.2	9.5	0.034
$P = 0.40$; $V_w^P = 97$ ml/loc													
650	629	400	282	85	−3.23	52.3	54.1	47.2	6.33	4.97	83.4	55.7	0.203
750	742	500	395	124	−1.12	45.3	45.8	37.5	4.55	3.17	47.9	25.4	0.139
850	851	600	504	158	+0.14	40.0	39.9	31.2	3.56	2.42	31.2	15.2	0.109
950	958	700	611	191	+0.83	35.8	35.5	26.8	2.95	2.03	22.2	10.5	0.090
1050	1063	800	715	223	+1.19	32.4	32.0	23.6	2.54	1.79	16.8	7.9	0.077
1150	1166	900	819	254	+1.38	29.6	29.2	21.1	2.26	1.63	13.2	6.3	0.068
$P = 0.60$; $V_w^P = 146$ ml/loc													
650	623	400	227	85	−4.23	52.3	54.6	41.6	6.33	3.83	83.4	35.5	0.304
750	744	500	348	124	−0.84	45.3	45.7	30.3	4.55	2.34	47.9	14.1	0.209
850	858	600	462	158	+0.91	40.0	39.6	24.2	3.56	1.83	31.2	8.4	0.163
950	967	700	571	191	+1.78	35.8	35.2	20.3	2.95	1.59	22.2	5.9	0.135
1050	1073	800	678	223	+2.20	32.4	31.7	17.5	2.54	1.45	16.8	4.5	0.116
$P = 0.80$; $V_w^P = 194$ ml/loc													
650	623	400	179	85	−4.19	52.3	54.6	29.7	6.33	2.28	83.4	13.4	0.406
750	752	500	308	124	+0.25	45.3	45.2	19.0	4.55	1.52	47.9	5.2	0.278

Table 1 *continued*

V_1^I $h = 0$	V_2^T	V_1	V_2	ΣQ	$\Delta V/V$	MC_1	MC_2	C_s	f_1	f_2	O_1	O_2	$h \cdot f_\Sigma$
$P = 0.20$; $V_w^P = 49$ ml/loc													
650	661	400	362	85	+1.62	52.3	51.5	48.4	6.33	5.25	83.4	61.2	0
750	769	500	471	124	+2.59	45.3	44.2	40.5	4.55	3.65	47.9	32.6	0
850	876	600	577	158	+3.02	40.0	38.8	35.0	3.56	2.85	31.2	20.8	0
950	980	700	681	191	+3.15	35.8	34.7	30.9	2.95	2.39	22.2	14.8	0
1050	1083	800	784	223	+3.14	32.4	31.4	27.6	2.54	2.09	16.8	11.2	0
1150	1185	900	887	254	+3.05	29.6	28.7	25.0	2.26	1.89	13.2	9.0	0
$P = 0.40$; $V_w^P = 97$ ml/loc													
650	677	400	330	85	+4.12	52.3	50.2	42.5	6.33	3.99	83.4	38.3	0
750	795	500	448	124	+6.03	45.3	42.8	34.1	4.55	2.74	47.9	19.3	0
850	907	600	560	158	+6.70	40.0	37.5	28.7	3.56	2.19	31.2	12.4	0
950	1015	700	668	191	+6.80	35.8	33.5	25.0	2.95	1.88	22.2	8.9	0
1050	1120	800	773	223	+6.65	32.4	30.4	22.1	2.54	1.70	16.8	6.9	0
1150	1223	900	876	254	+6.39	29.6	27.8	19.9	2.26	1.57	13.2	5.7	0
$P = 0.60$; $V_w^P = 146$ ml/loc													
650	702	400	306	85	+7.94	52.3	48.5	33.5	6.33	2.67	83.4	18.4	0
750	828	500	433	124	+10.42	45.3	41.1	25.5	4.55	1.93	47.9	9.4	0
850	944	600	548	158	+11.02	40.0	36.0	21.0	3.56	1.63	31.2	6.3	0
950	1053	700	658	191	+10.89	35.8	32.3	17.9	2.95	1.47	22.2	4.7	0
$P = 0.80$; $V_w^P = 194$ ml/loc													
650	737	400	293	85	+13.33	52.3	46.2	19.9	6.33	1.57	83.4	5.6	0

The Table was constructed for a small, selected range of h, P and V^T values (*see* Materials and Methods), to illustrate its use. C_P was set at 69 g/dl and C_{sat} at 17 g/dl. The computations satisfied $C_s \geq C_{sat}$. This determined the decreasing range of V_1^T values obtained at the higher polymer fractions. The meaning and units of the column headings are as follows (*see* Glossary for extended definitions): subindexes 1 and 2 indicate values before and after polymerization; V_w^P is the volume of polymer-associated water, in ml/340 g Hb; V^T and V are total cell volume and volume of cytosolic cell water (polymer water excluded), respectively, in ml/340 g Hb; ΣQ is the total amount of small cell osmoticants, in imosmol/340 g Hb; $\Delta V/V$ is the percent change in total cell volume induced by polymerization; MC represents MCHC, in g/dl; C_s is the concentration of residual soluble Hb in the cytosol, in g/dl; f is the osmotic coefficient of soluble Hb in the cytosol, in iosmol/mol; O represents the iosmolality of soluble Hb in the cytosol, in imosmolal; and $h \cdot f_\Sigma$ is the fraction of small cell solutes contained within polymer water.

mer fraction (Fig. 2, panel *4*). Thus the more dehydrated the SS cells are before polymerization, the more they will dehydrate proportionately on polymerization; in the densest cells, the volume reduction may exceed 10%.

The additional osmotic effects predicted as a function of polymer fraction include: (*i*) decrease in cytosolic water and increase in polymer water (Fig. 2, panel *3*); the cross-over points indicate the polymer fraction at which cells with different initial MCHCs will contain comparable volumes of water in cytosol and polymer, (*ii*) decreases in soluble Hb concentration in the cytosol (Fig. 2, panel *1*), osmotic coefficient of Hb (Table 1), and in iosmolality of Hb (Fig. 2, panel *2*), and (*iii*) the increase in the fraction of total low MW cell osmoticants within polymer water (Fig. 2, panel *5*).

Though these effects are qualitatively similar in cells with different prepolymerization volumes, the shape of the curves and the slopes vary considerably with initial MCHC (except those for cytosolic and polymer water in Fig. 2, panel *3*). In general, most changes are steeper in dehydrated cells (higher MCHC) when polymer fractions are above 40%.

A detailed analysis of some specific examples from Table 1 ($h = 1$) may help describe the osmotic effects of polymerization in cells with different initial MCHCs, and point out the components which affect the overall osmotic response.

In cells with an initial "MCHC" ([Hb]) of ≈36 g/dl, 60% polymerization reduces the osmotic contribution of Hb from about ≈22 to ≈7 imosmolal, with a ≈4% reduction in cell volume (Table 1 and Figs. 1–3), whereas with an initial MCHC of 45 g/dl, the reduction in soluble Hb iosmolality is from ≈48 to about 20 imosmolal, with over 8% decrease in cell volume. At any given intermediate oxygen saturation at which polymerization occurs, however, the differences in volume shifts between cells with such different initial MCHCs will be more dramatic, since more polymer would form within the higher MCHC cells. Comparisons at a given (low) pO_2 would further exaggerate these differences, since the more dehydrated SS cells exhibit a lower oxygen affinity.

Table 2. Effect of oxygenation on the density distribution of sickle cell anemia red cells from freshly drawn venous blood

Donor	δ	Fresh venous blood	After oxygenation	% Change
		% Cells with density > δ		
	1.106	23.3 ± 0.1	18.0 ± 0.5	−23
1	1.118	7.8 ± 0.4	5.2 ± 0.2	−33
	1.125	5.9 ± 0.1	3.8 ± 0.1	−36
	1.106	50.8 ± 0.3	48.8 ± 0.1	−4
2	1.118	34.6 ± 0.3	30.2 ± 0.2	−13
	1.125	23.9 ± 0.1	18.5 ± 0.2	−23
	1.106	25.7 ± 0.3	21.7 ± 0.4	−16
3	1.118	14.5 ± 0.2	11.8 ± 0.1	−19
	1.125	10.0 ± 0.0	6.7 ± 0.1	−33
	1.106	16.3 ± 0.2	13.3 ± 0.0	−18
4	1.118	9.2 ± 0.1	7.7 ± 0.0	−16
	1.125	6.7 ± 0.1	5.4 ± 0.1	−19
	1.106	38.2 ± 0.3	33.3 ± 0.6	−13
5	1.118	19.9 ± 0.2	17.1 ± 0.1	−14
	1.125	19.3 ± 0.3	16.2 ± 0.1	−16
	1.106	38.0 ± 0.0	30.9 ± 0.1	−19
6	1.118	24.8 ± 0.1	18.8 ± 0.1	−24
	1.125	24.9 ± 0.3	18.6 ± 0.1	−25

The percent cells with density > δ was measured in triplicate and the results are reported as the mean ± SEM.

To examine the basis of the fall in Hb iosmolality due to polymer formation, let us consider further the example of the cell with an initial MCHC of 45 g/dl. The analysis predicts (Table 1) that with 60% polymerization, total Hb iosmolality falls from 48 to 20 imosmolal. The two components in this fall are the soluble Hb concentration in the cytosol (from MC_i to C_s), which changes from ≈45 to ≈35 g/dl, and the osmotic coefficient of Hb (f), which drops from 4.6 to 2.8 iosmol/mol. This means that the reduction in Hb osmolality on polymerization results from the sum of two components: direct removal of osmotic particles by polymer formation and dilution of residual soluble Hb, equivalent to a reduction in osmotic coefficient. Direct support for dilution of residual soluble Hb upon polymerization was provided by earlier results of Eisinger, Flores and Bookchin [14] who showed that quenching of fluorescent probes embedded in the lipid bilayer by adjacent cytosolic Hb is reduced after deoxygenation of SS red cells.

Comparison of iosmolalities of Hb (O_i) and of non-Hb solutes (ΣQ) throughout Table 1 shows that the osmotic contribution of low MW cell osmoticants falls from ≈254 to ≈85 imosmol/340 g Hb, whereas that of Hb increases from ≈13 to ≈83 imosmolal as the MCHC increases from ≈30, its value in reticulocyte-rich density fractions, to ≈52 g/dl, the value which corresponds to the densest (irreversibly sickled) cell fraction. Thus, in cells dehydrated by salt loss, as with SS cells (*D*3), Hb becomes a major osmoticant, and therefore, the polymerization-induced volume shifts, all linked to changes in Hb iosmolality, will generally be larger in dehydrated than in normal volume SS cells. The computations reported in Table 1 and Fig. 2 were based on the assumption that C_P is 69 g/dl. It will be seen below that exceptions to the above patterns occur with different C_P values.

Role of Polymer Hydration on the Osmotic Effects of Polymerization

Our preliminary measurements of C_P using a new method [4] gave a value of 59.1 ± 0.9 g/dl, nearly 20% below the previous estimates of ≈69 g/dl. Those results prompted us to assess the role of polymer-associated water in the cell volume changes accompanying Hb polymerization by comparing predictions from models with different values of C_P.

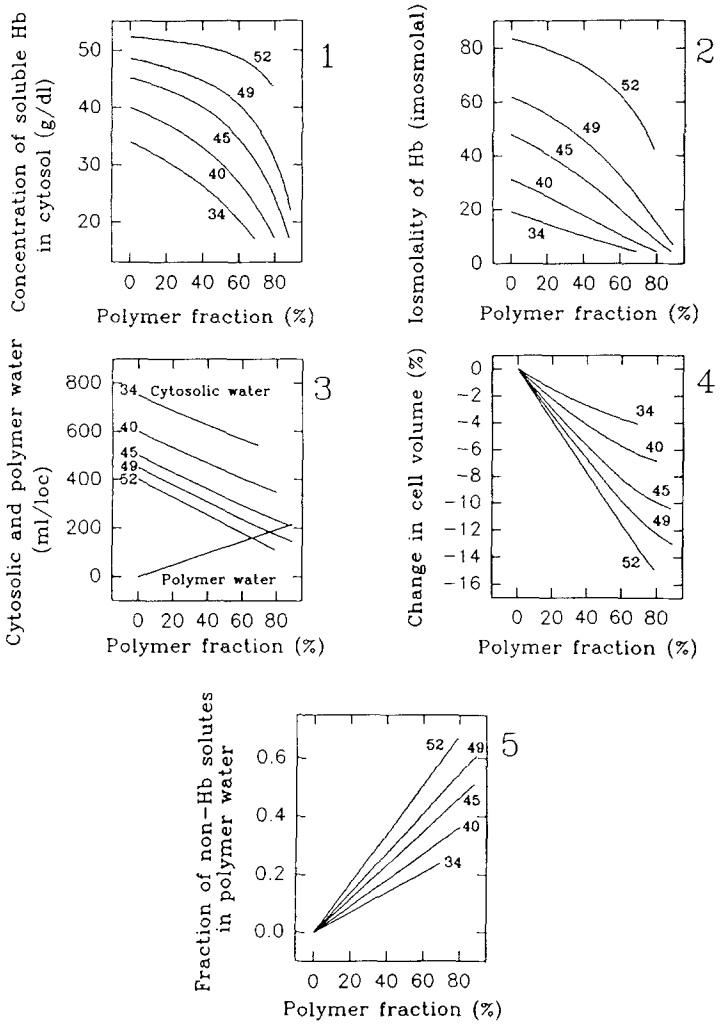

Fig. 2. Predicted changes in osmotic parameters of SS cells as a function of polymer fraction. Model with no exclusion of low MW osmoticants from polymer water ($h = 1$). Panels numbered from left to right and from top to bottom. Five curves within each panel represent the behavior of cells with the indicated initial MCHCs. Panel *1:* concentration of unpolymerized oxy- and deoxy-Hb S in cytosol. Panel *2:* iosmolality of unpolymerized Hb in cytosol. Panel *3:* volume of cytosolic and polymer-associated water; polymer water is determined by C_P (10.7 mM, or 69 g/dl, in this figure) and is independent of cell volume or MCHC. Panel *4:* percent change in total cell volume. The curves in this panel are the same as those represented by solid lines ($h = 1$) in Fig. 1. Panel *5:* fraction of low MW cell osmoticants within polymer-associated water, assuming osmotic equilibrium between cytosolic and polymer solutions; only low MW cell osmoticants make up iosmolality of polymer solution, whereas soluble Hb (oxy and deoxy), excluded from polymer water, contributes only to the iosmolality of the cytosol

We first compared predictions from a model with $C_P = 69$ g/dl (Table 1 and Fig. 2), with those from an unrealistic model (*not shown*) in which polymer water is ignored (equivalent to assuming that $C_P \approx C_H$, 136 g/dl, i.e., that the polymer is fully dehydrated). Both models predicted polymerization-induced shrinkage at all initial cell volumes, but the shrinkage was larger when $C_P \approx C_H$. Since both models now assume full osmotic equilibration of low MW solutes within the cell's water compartments, the basic difference between these two models is the inclusion in one of a polymer-associated water compartment which excludes only macromolecules (mainly soluble Hb). Thus, the lesser shrinkage predicted by the model with polymer-associated water must result from effects of exclusion of soluble Hb from that polymer water compartment. The higher the MCHC, the more substantial the difference between the two models, reflecting the fact that polymer-associated water comprises a larger fraction of the total cell water in the more dehydrated, deoxygenated SS cells.

We next compared the osmotic effects predicted by models with C_P values of 69 g/dl (Fig. 2, ≈48% water in polymer) and 56 g/dl (≈59% water in polymer), one of the lower experimental values in our studies [4] (Fig. 3). Compared with the model in which $C_P = 69$ g/dl (Fig. 2), the model in which $C_P = 56$ g/dl (Fig. 3) shows: (*i*) a cross-over point for polymer and cytosolic water (panel *3*) at lower polymer fractions, reflecting the larger relative volume of polymer water at the lower value of C_P; (*ii*) at initial MCHCs above ≈49 g/dl, residual soluble Hb becomes concentrated rather than diluted after polymerization (panel *1*); it may become even more concentrated than in the polymer (C_P); in the most dehydrated cells, its iosmolality may also rise sharply as the polymer fraction increases (panel *2*); (*iii*) at high polymer fractions, the proportion of the cells' low MW osmoticants in the polymer water compartment

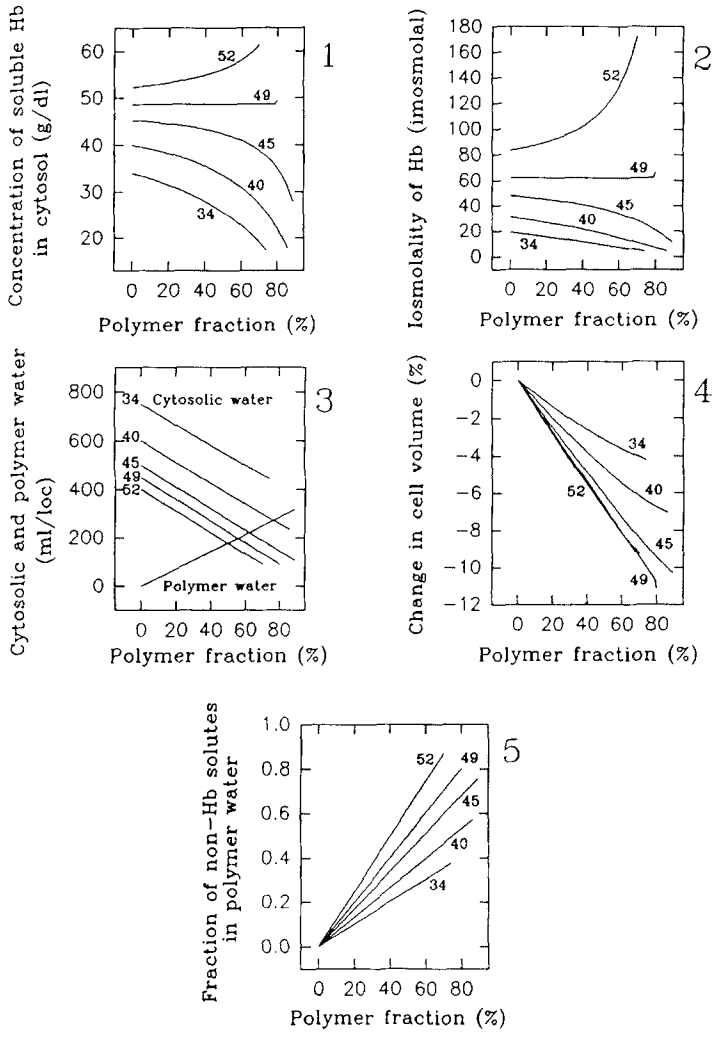

Fig. 3. Predicted changes in osmotic parameters of SS cells as a function of polymer fraction, assuming higher polymer hydration than in Fig. 2 (C_P = 8.7 mM, or 56 g/dl). Except for C_P, panel descriptions are as for Fig. 2

may reach 80% or more (panel 5); and (*iv*) polymerization-induced cell dehydration tends to reach a maximum at MCHCs of about 49 g/dl (panel 4). This analysis indicates that, if $C_P \approx 56$ g/dl, then in cells with a high initial MCHC the Hb excluded from the polymer-associated water is squeezed into residual cytosol at a higher concentration, and with a higher osmotic coefficient, than before polymerization. The low MW solutes, on the other hand, become more concentrated in polymer water than in the cytosol, balancing the osmotic effect of polymer-excluded Hb. Despite the opposite changes in Hb iosmolality, models with high or low C_P values predict overall cell shrinkage at all MCHCs. These results suggest a critical experiment to test whether polymer hydration corresponds to C_P values nearer to 56 than to 69 g/dl. If $C_P \approx 56$ g/dl, then with SS cells dehydrated by salt loss to MCHCs > 50 g/dl, full deoxygenation should increase the quenching by cytosolic Hb of fluorescent probes embedded in the bilayer, reversing the trend observed at lower MCHCs [14].

The curves reporting osmotic parameter changes for cells with initial MCHC of 52 in Fig. 2 (C_P = 69 g/dl) and with initial MCHCs of 49 and 52 in Fig. 3 (C_P = 56.1 g/dl), are defined up to polymer fractions lower than those of cells with initial MCHCs of 45 g/dl or less. In these conditions C_s stays well above C_{sat}; the effect is thus unrelated to limitations on P_{max} due to C_{sat}. The reason for the apparent reduced P_{max} at the high MCHCs is the lack of a real solution for V_2^I in Eq. (13). This simply means that one or more of the assumptions applied in the derivation of Eq. (13), or some of the parameter values used for the computations, must have been or have become inadequate to describe the osmotic equilibria prevailing in such highly dehydrated cells.

Clear candidates for misbehavior at high Hb

concentrations are Eqs. (2) and (11) which may need additional, or corrected, virial coefficients when the soluble Hb concentration exceeds 35 g/dl [18]. These equations have not yet been refined for the range of Hb concentrations above 45–49 g/dl. In red cells dehydrated by salt loss, ionic strength changes much less than in cells exposed to hypertonic shrinkage. The osmotic anomalies considered here are therefore unlikely to be due to changes in ionic strength [1, 38]. A search for refined virial equations is outside the scope of the present analysis.

Discussion

The osmotic effects of deoxy-Hb S polymerization were analyzed with alternative models, which assumed that low molecular weight non-Hb osmoticants either are, or are not, excluded from polymer-associated water. To test the opposite predictions of exclusion and nonexclusion models experimentally, we measured the oxygenation-induced density shifts of SS cells from fresh venous blood and obtained support for the nonexclusion model. The analyses and results indicate that SS cells would shrink on deoxygenation and swell on reoxygenation, and that the size of the volume shifts in dehydrated SS cells may be far larger than had hitherto been suspected.

From the analysis, we can identify several interrelated factors that determine the extent of the cell's volume change on polymerization or depolymerization: (*i*) the direct change in osmotic particle concentration due to polymer formation or breakdown, (*ii*) the extent to which soluble Hb and non-Hb osmoticants are excluded from polymer-associated water, (*iii*) the change in polymer fraction with total cell Hb concentration, (*iv*) the change in osmotic coefficient upon dilution or concentration of the soluble Hb, and (*v*) the proportionate contribution of Hb to the total cell osmoticants (which is relatively high in dense SS cells which have become dehydrated by salt loss). The last factor above, acting together with the two preceding factors, considerably enhances the volume response of dehydrated SS cells. We will now discuss some implications of the present analysis.

Consequences of Assuming Osmotic Equilibrium between Cytosolic and Polymer Water Compartments

Preliminary evidence indicating that sorbitol and small ions fully equilibrate in the polymer water compartment, whereas macromolecules remain excluded [4] suggests that equality of the chemical potential of water in the polymer and the cytosol is attained together with osmotic equilibration. Analysis of such an osmotic equilibrium exposes unexpected properties of the polymer boundaries and possible metabolic effects. If cytosolic macromolecules are excluded from polymer water, their osmotic contribution in the cytosol must be balanced by a higher concentration of nonexcluded solutes in the polymer water. This must generate a Donnan distribution with stationary diffusion and potential gradients across the exclusion boundary on the polymer surfaces. Although the experimental results rule out global exclusion of non-Hb solutes, they are not sufficiently quantitative to rule out partial exclusion. Any selective solute exclusion would also cause additional composition differences between polymer water and cytosol and associated diffusion and potential gradients. All such effects would be larger in dehydrated SS cells, where polymer water becomes a substantial fraction of the cell's total water. Another possible anomaly in dehydrated SS cells could result from the exclusion of enzymes from the polymer water: their consequent increased cytosolic concentration, and the reduced access-time to their substrates, could alter the operation of the cell's metabolic and repair processes in unforseen ways.

Implications for the Statistical Mechanical Analysis of Concentrated Protein Solutions

Statistical mechanical analysis of the behavior of rigid, noninteractive spheres, using the virial expansion technique [20, 34, 36], adequately predicts the virial coefficients needed to describe the nonideal behavior of Hb in sedimentation [13, 36] and in osmotic equilibration experiments [1, 3, 11, 12, 28] when the Hb concentration is below ≈35 g/dl. Within this Hb concentration range, then, Hb molecules in aqueous solution behave like free bouncing "billiard balls." At higher Hb concentrations, the virial coefficients used in Eqs. (2) and (11) systematically underestimate the osmotic effects observed [18], suggesting that in red cells dehydrated by salt loss (relatively constant ionic strength), interactions at shorter intermolecular distances may generate further departures from nonideality, requiring additional coefficients.

An experimental model for cell dehydration by salt loss can be approximated using ionophores for K permeabilization of normal red cells, which then dehydrate with loss of KCl. The increased osmotic value of concentrated hemoglobin causes more KCl than water to be displaced from the cells, with conse-

quent increase in the overall osmolality of the cell suspension [18]. A statistical mechanical interpretation of this effect must account for an increased partial pressure (and hence activity coefficient) of the low molecular weight solutes in the cell's domain of increasing protein concentration, to explain what drives a hypertonic effluent out of the cells. A similar interpretation would have to apply to the unequal distribution of low molecular weight solutes in cytosolic and polymer water, as suggested by our analysis.

Possible Hemodynamic Effects of HB S Polymerization

Because of the very low oxygen affinity of dense SS cells, they are largely deoxygenated at the oxygen tensions in the capillary circulation, and a substantial fraction of their Hb is polymerized. Under those conditions, they would be partially shrunken compared with their volume when fully oxygenated and free of polymer, as they may be observed under laboratory conditions. The shrinkage resulting from polymerization would tend to decrease cell deformability (as compared with the oxygenated cells), with consequent reduction in local flow. Stasis would lead to more complete deoxygenation of the cells and increased polymer fraction, with further shrinkage and rigidity, thereby increasing the risk of microinfarction.

Possible Physiological Roles of the Osmotic Changes Generated by Protein Polymerization-Depolymerization

The analysis presented here, although specifically focused on Hb S and SS cells, applies to protein-dependent osmotic effects in any cell. Reversible polymerization reactions occur in most cells, and mediate many essential cell functions [19]. In the light of the present analysis, possible physiological roles of protein-dependent osmotic shifts may be worth exploring, such as in the swelling of secretory granules after fusion [43], or in the generation of local osmotic gradients for water transport.

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