ELSEVIER

Biochimica et Biophysica Acta 1192 (1994) 223–233

BBA Biochimica et Biophysica Acta

Morphology and volume alterations of human erythrocytes caused by the anion transporter inhibitors, DIDS and *p*-azidobenzylphlorizin

Michael E. Blank [a,d], Daniel M. Hoefner [a,b], Donald F. Diedrich [a,c,*]

[a] *Center of Membrane Sciences, University of Kentucky, Lexington, KY, USA*
[b] *Graduate Center for Toxicology, University of Kentucky College of Medicine, Lexington, KY, USA*
[c] *Department of Pharmacology, University of Kentucky College of Medicine, Lexington, KY, USA*
[d] *Department of Physiology, University of Hamburg, Hamburg, Germany*

(Received 1 September 1993)

Abstract

p-Azidobenzylphlorizin (*p*-AzBPhz) is a potential photoaffinity labeling agent for the anion and glucose transporters in human RBCs. In the absence of light and at the same low concentrations which block these transport processes (only 1–2 million molecules bound/cell), this impermeable membrane probe produces rapid morphological and volume alterations. This high-affinity activity, called phase 1, can be rapidly and completely reversed by simply diluting the azide-treated cell suspension. Phase 2 effects, including formation of cells with multiple, long spicules (stage 3/4 echinocytes), followed by an influx of salt and water with eventual lysis, occur at two log units higher concentration by a different mechanism, probably by intercalating into and selectively expanding the outer lipid monolayer. Light scattering, electronic cell sizing, microhematocrit measurements and scanning electron microscopy have been employed to compare the effects of the azide and the anion transport inhibitor, DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonate), on red cells. DIDS produced only those changes analogous to the azide's low dose phase 1 action; cells swell, lose the ability to scatter 800 nm light and undergo a limited shape change (comparable to stage 1 echinocytosis). The mechanism by which the two ligands perturb the membrane is additive, suggesting that a Band 3-mediated transmembrane signaling is involved which leads to altered cytoskeleton dynamics.

Key words: Echinocyte; Cytoskeleton; Band 3; Erythrocyte membrane; Cell shape; Cell volume; DIDS

1. Introduction

The membrane impermeable 5′-*p*-azidobenzyl derivative of the phenolic glucoside, phlorizin, (*p*-AzBPhz), was one of several agents synthesized to photoaffinity label the Na^+/glucose co-transporter [1]. Although its minimal affinity for the renal [2] and intestinal (unpublished) sugar transport mechanisms limited its use as a probe in these systems, it is unexpectedly potent as a competitive inhibitor of the Na^+-independent sugar transporter in human erythrocytes [1]. Even more surprising is its high affinity for the anion transporter in this membrane. The ligand blocks chloride equilibrium exchange at nominal concentrations less than 2 μM [1,3]. Results from these inhibition studies and binding experiments with the tritiated azide indicated that half-maximal inhibition of the anion exchanger was attained when less than a million azide molecules were bound to each RBC membrane. When about 2.6 million molecules were bound, half of the glucose transport capacity was also blocked [3,4].

While attempting to determine the optimal conditions for photolabeling these two RBC transport systems, we noticed that *p*-AzBPhz caused cell swelling in isosmotic media even before photoactivation [5]. In a follow-up study involving light scattering measurements, scanning electron microscopy and electronic cell sizing [4], we suggested that the ligand had two distinct, dose-dependent actions on the membrane. High concentrations (5–60 μM) caused loss of mem-

* Corresponding author at University of Kentucky College of Medicine, Department of Pharmacology, Lexington, KY 40506-0084, USA. Fax: +1 (606) 2581981.

Abbreviations: *p*-AzBPhz, *p*-azidobenzyl-5′-phlorizin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

0005-2736/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0005-2736(94)00099-B

brane integrity and hemolysis within 30 min – expected for an amphoteric ligand. However, at two orders of magnitude lower concentration, when about 1 million molecules were bound per cell, and when the anion (and sugar) transporters were inhibited, the ligand also induced cell morphology changes, a decreased 800 nm light scattering ability, and a small water (but not cation) influx. The cell's transition to a new, stable steady state was shown by these methods to be complete within 1–2 min, although a video imagining technique indicated that the change occurs within seconds [1]. The ligand appeared to be bound superficially rather than being embedded in the lipid bilayer since addition of drug-free buffer reversed these low-dose effects just as rapidly. This suspicion was supported by the results of an electron spin resonance study [3] in which a lipid-specific spin label was inserted into the cell membrane. After the addition of low levels of *p*-AzBPhz to these cells, no change in the order and motion of the spin label monitoring the outer monolayer could be detected.

These observations intensified our interest in determining the mechanism of the azide's bipartite effects. The aim of the present work was to determine whether the morphology and volume changes induced by the lowest ligand doses could be a consequence of the inhibitory action on the anion transporter. In this case, we reasoned that other well-established Band 3 antagonists should cause similar responses. DIDS was the agent chosen to test this idea because a structural similarity between phloretin (and by inference, *p*-AzBPhz) and the disulfonic acid stilbenes has already been described. The two ligands appear to share a common binding site on the anion exchanger [6,7] and both influence water flux [8]. However, other workers dispute this relationship [9] and cite earlier data indicating that phloretin, and even the more polar phlorizin, are localized in the membrane phospholipid layer where they modify the dipole potential at both interfaces [10–14]. These divergent views can be unified if one takes into account that phloretin and phlorizin possess an unusually acidic 4'-phenolic group, $pK_a = 7.2$ [1,14,15]. It can then be argued that the protonated, uncharged molecules could interact with lipid [8,13,15], whereas the anionic species are preferentially bound to Band 3 [6,8]. *p*-AzBPhz's bimodal action can be interpreted in this context, which leads to the following working hypothesis: Loss of membrane integrity, formation of highly spiculated cell structures and eventual lysis occurs when a sufficient number of uncharged azide molecules have accumulated in the outer lipid monolayer. Its reversible, rapid action, that results in membrane blistering, water influx and increased flexibility (to be reported elsewhere) is due to its high affinity, ionic binding to the anion transporter.

It would not be surprising if *p*-AzBPhz, as it engages its Band 3 receptor, would modify either the self-association of the tetrameric form of the protein or its tertiary structure [16], including its cytoplasmic domain. Either of these transformations might be expected to influence cytoskeletal organization [17,18]. There are at least two precedents for this kind of transmembrane signaling when DIDS interacts with Band 3: (i) hemoglobin's association with the transporter's intracellular terminus becomes modified [19], and (ii) the cytoskeletal network is rearranged so that the alkaline extractability of spectrin and ankyrin is altered [20]. In the current work, the effects of DIDS and low dose *p*-AzBPhz on RBC morphology and volume have been compared. We conclude that these ligands influence the erythrocyte shape and volume by an identical mechanism as they interact with the Band 3 protein.

2. Experimental procedures

2.1. Materials

p-AzBPhz was synthesized from the corresponding amine [1]. Sigma specified that the DIDS we used was 80–90% pure. Na^+PBS and K^+PBS buffers were, respectively, 154 mM sodium or potassium chloride plus 15 mM of the corresponding phosphate, final pH 7.4; 320 mosM, verified by osmometer. The solutions were usually passed through 0.2 μm bacterial filters. A 154 mM, low sodium (about 20 mM) buffer consisted of *N*-methylglucamine chloride plus 15 mM phosphate (pH 7.4).

2.2. Erythrocyte preparation

Blood was obtained from five hematologically normal volunteers into Vacutainer (K^+ EDTA) tubes. The red cells were isolated within an hour of collection by low speed centrifugation at room temperature. After removing plasma and buffy coat, the cells were washed at 20°C only twice (to avoid spontaneous echinocytosis) with 40 volumes of the buffer to be used in the experiment. When volume was to be altered, cells were

[1] We have recently examined the kinetics of this phenomenon using a quantitative video image morphometry technique. Cell biconcavity was lost and cell flattening occurred within 2 s of low level drug addition. If the azide dose was slightly increased, membrane bulges were formed as the cell appeared to swell. Steady states were reached within 15 s. Complete restoration to normal sized discocytes within 1–2 s occurred by simply washing the cells free of drug (Hoefner, D.M., Davis, B.M. and Diedrich, D.F. (1992) Toxicologist 12, 184 (Abstr. No. 661)). In a subsequent study, both DIDS and *p*-AzBPhz increased the flexibility of normal and sickle RBCs, measured as an increase in microfilterability.

suspended in osmotically adjusted (with water or sucrose) Na^+PBS buffers. For the light scattering experiments, cell suspensions were normally adjusted to 7.5 million cells/ml (about 0.07% hematocrit), verified with the Coulter Counter (model Z_{BI}).

2.3. p-AzBPhz and DIDS treatment of cells

Except when indicated, experiments with the azide and DIDS were conducted in subdued light within an hour of RBC isolation. During some occasional, lengthy procedures (6 h), the inherent change in MCV and light scattering capacity of untreated cells was under 3%. Aliquots from stock, ethanolic *p*-AzBPhz solutions were added to buffers a few minutes before use; the same volume of each drug solution (or vehicle) was added to cell suspensions so that all contained ethanol at the same final concentration of 6 mM (0.03%). Alcohol must reach 0.05% before a detectable decrease in light scattering signal occurs. Stock solutions of DIDS in Na^+PBS were prepared shortly before an experiment and were kept in the dark until used. Normally, the effect of DIDS was measured at room temperature 30 min after addition to red cells.

2.4. Examination of cell morphology

Aliquots of control or drug-treated cell suspensions were mixed at 20°C with 10 volumes of 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cell fixation at this aldehyde concentration was rapid enough to prevent the reversal of *p*-AzBPhz effects due to dilution. No echinocytosis artifacts occurred under these conditions [4]. The fixed cells were then collected on Nuclepore polycarbonate filters, rinsed with buffer and processed in situ with a gradient ethanol dehydration and critical point drying in CO_2. The Au/Pd sputter-coated sample was observed at 10–15 kV with a JEOL 35 CF scanning electron microscope.

2.5. Light scattering assay

An Aminco DW 2a recording spectrophotometer fitted with the Total Fluorescence Accessory was used to measure the effects of erythrocyte shape and volume changes. In the split beam mode, the instrument registers differences in 90° scattering of 800 nm incident light produced by irradiating suspensions of untreated control and ligand-modified cells at 20 ± 1°C (maintained with a thermostated cuvette holder). After adding identical 3 ml volumes of a 0.07% cell suspension to the reference and sample cuvettes, the light beams were balanced and recorded on chart paper as 0% T difference. At zero time, 0.5 ml of vehicle or ligand was mixed with the cells using an automatic pipette; nothing was added to the reference suspension. After closing the sample compartment shutter within 5 s, pen deflections (in cm) were recorded for as long as an hour. When buffer only was added to the sample, a 6–7 cm decrease in scatter was observed due to dilution. An increase in scattering units was recorded when light deflection was even less than attributable to dilution, representing the cell's decreased ability to scatter light.

2.6. Electronic cell counting and sizing

The Coulter Counter with Channelyzer, model C-1000, was used to count the cell population and to estimate relative volume changes. These parameters were determined from current impedance across a 100 μm aperture through which the cells pass. The Channelyzer produces a graphic and numeric size analysis of a RBC population distribution, registered as a Channelyzer number that is proportional to MCV. The instrument was calibrated with latex microspheres and Coulter's 4C PLUS (normal, abnormal high and abnormal low) red cells were used as controls under low count instrument settings. Coincident counting was minimized by examining only low density cell suspensions ($\sim$ 15 000 cells/ml). *p*-AzBPhz-treated cells were surveyed at 4 min, after they had attained steady state [4]. It was important to add azide to the counting vessel buffer at a concentration equivalent to that in the RBC sample to prevent the rapid reversal of azide binding.

2.7. Photolysis

The procedure used to measure light scattering was adapted to photolyze membrane-bound *p*-AzBPhz. After recording the ligand's effect at 800 nm, the incident light was adjusted to 255 nm and both the control and treated cells were irradiated at this azide-activating wavelength (1 cm light path). At varying intervals, 20-μl samples of the suspensions were added to the Coulter counting reservoir buffer containing either vehicle or azide at the concentration being examined. Cell count and volume profiles were then determined.

2.8. Microhematocrit and extracellular space measurements

In order to obtain sufficient cells for this analysis, 1% suspensions were tested. Four min after azide addition or after 30 min with DIDS, cells were centrifuged at very low speed to about a 30% hematocrit; 150 000 dpm of an extracellular space marker ([^{3}H]inulin) was added at this stage. Triplicate aliquots of cells were then centrifuged at $12\,000 \times g$ for 15 min in a micro-capillary centrifuge and the packed cell volume

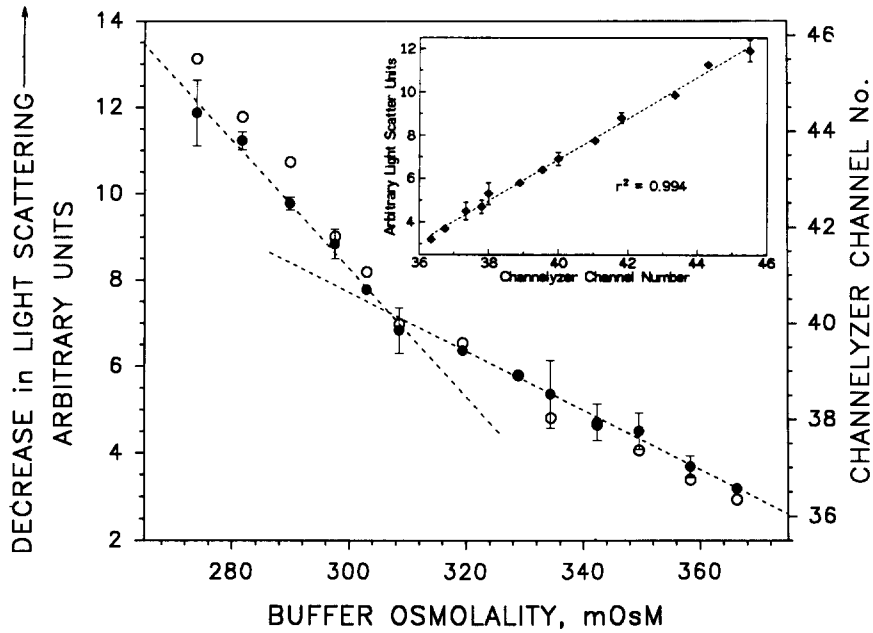

Fig. 1. Correlation of cell volume with light scattering capacity and Coulter Channelyzer number. Cell volume was altered by adding water or sucrose to RBCs in 320 mosM PBS buffer; an osmometer was used for calibration. Swollen cells lost capacity to deflect 800 nm light, measured as arbitrary light scattering units (•); osmotically contracted cells scattered more light. Two dotted regression lines of these mean values above and below 310 mosM are shown. Electronic cell sizing measurements are given as Channelyzer channel number (○) representing the mean size distribution of the cells. Channel numbers are convertible to approximate cell volume: [Channel No. +20 (BCT)] × 1.482 (TF), where BCT is a base channel threshold, an operator defined setting that excludes low-end noise, and TF is the threshold factor generated from instrument calibration using latex microbeads. Thus, for control cells in 320 mosM PBS, the mean calculated MCV was 89 fl [(40.1 + 20)(1.482)], corresponding to a baseline light scattering of 6.5 units. Values are means ± S.D. of duplicate observations from three different donors. The inset shows the degree of correlation between the two measured parameters.

was measured with calipers using a magnifying glass [2]. Capillary tubes were cut at the cell/supernate interface, and after blotting traces of supernatant from the cell column, the packed RBCs were extruded with a close fitting glass rod, and lysed in a volume of water. Extracellular space was determined by measuring radioactivity in the packed cells and supernatants. Protosol (Dupont/NEN Research Products) was used as tissue solubilizer and peroxide as decolorizer according to the manufacturer's instructions. Hemoglobin was measured at 540 nm as cyanmethemoglobin.

2.9. Statistics

Means ±S.D. are reported for all independent experiments. The Student's *t*-test was used to judge the level of significance.

[2] Duplicate or triplicate measurements were made with Yankee brand tubes (Becton, Dickenson) which were of superior uniformity compared to other brands. They were calibrated to verify their 1.1 mm i.d. by measuring the column length (±0.1 mm) of tritiated water in relation to the amount of radioactivity.

3. Results

3.1. Correlation of RBC volume, light scattering ability and electronic sizing measurements

Fig. 1 shows the correlation of the Coulter channelyzer signal with the light scattering capacity of cells whose volume was altered by modifying the medium osmolarity. An increase in cell volume in hypotonic media was detected as both an increase in Channelyzer number and, as expected for swollen cells, a decreased ability to scatter light. Opposite effects were observed when cells were made to shrink in sucrose-containing hypertonic buffer. When the values for both signals were plotted over the osmolarity range examined, very similar non-linear curves were obtained with inflections occurring at about 310 mosM. The decreased slope at higher osmolarities is attributable to the curvilinear increase in hemoglobin's osmotic (activity) coefficient as its concentration increases [21,22]. The inset in Fig. 1 indicates the excellent comparison between the two assay methods with negligible random error. These data support the use of the Channelyzer and light scattering as a means to estimate the degree of ligand-induced changes in cell volume.

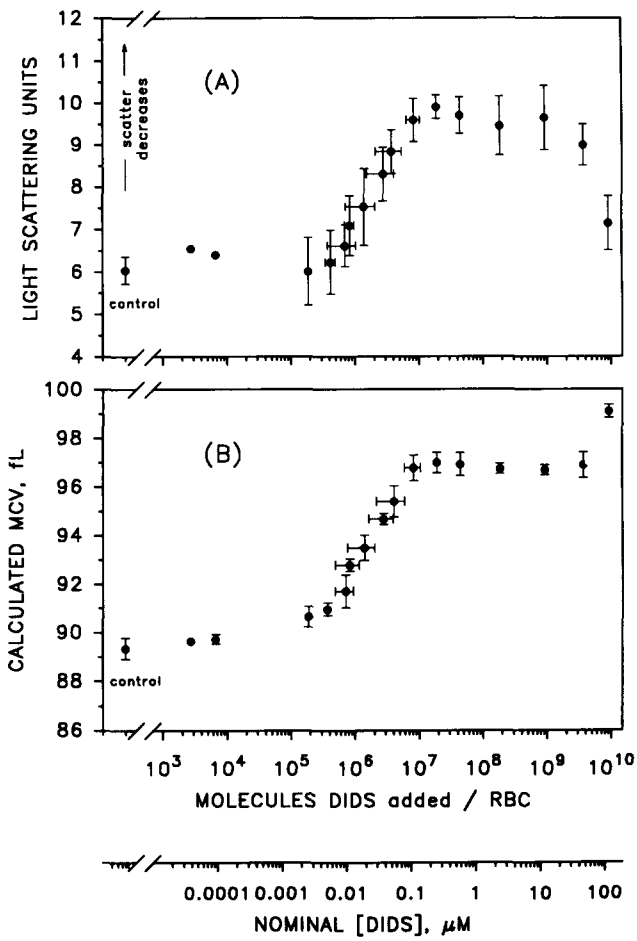

Fig. 2. DIDS alters light scattering and electronic sizing measurements. Panel A shows the dose-dependent decrease in 800 nm light scattering ability of 0.07% suspension of cells in Na^+ PBS treated with DIDS for 30 min at 22°C; scattering index values are means ± S.D. of four or five experiments, two or three replicates each. At 0.1 μM, when only about 5 million DIDS molecules/cell had been added, light scattering ability was maximally decreased and the cells were converted to stage 1 echinocytes. A corresponding cell swelling of about 10% was measured (Panel B). No further changes were observable over a concentration increase of nearly three log units. DIDS binding was covalent; 5 min after drug exposure the increase in MCV signal was no longer reversible upon dilution.

3.2. Light scattering and MCV measurements of DIDS-treated RBCs

Results in Fig. 2 show that DIDS produces the same morphological and volume changes in red cells as those we observed in an early study [4] with the lowest doses of *p*-AzBPhz. The initial effect was a very rapid and pronounced cell flattening and loss of biconcavity which was detectable with our video imaging method when as few as 0.5 million DIDS molecules/ RBC were added (data not shown). Over the range 0.005–0.06 μM, the stilbene converted these smooth-surfaced, flattened disks into cells with 25–30 blunt, blister-like extrusions. They resembled the stage 1 echinocytes as described by Brecher and Bessis [23] and appeared to be identical in form as those produced by about 5–10-times greater concentration of *p*-AzBPhz (see our previously published Fig. 4 II and III micrographs in Ref. [4]). With this morphology change, the cells' ability to scatter

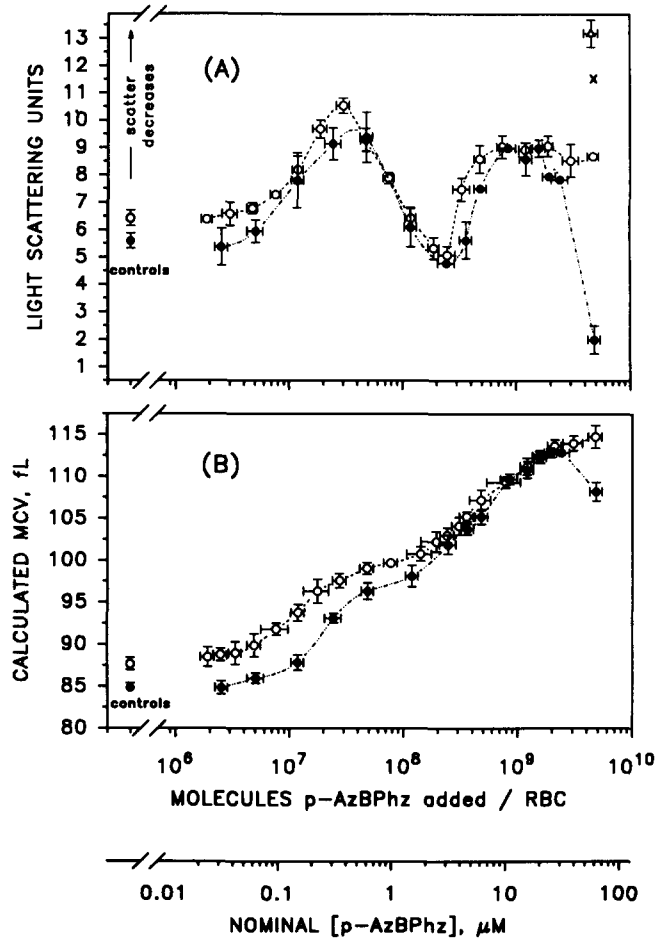

Fig. 3. RBC light scattering ability and volume alteration with *p*-AzBPhz. (Panel A) Scattering index values are means (±S.D.) of light deflection measurements taken at the new steady state, 4 min after azide addition to cells in Na^+ PBS (○), duplicate or triplicate observations ($n = 3$–6 experiments); or in *N*-methylglucamine chloride buffer (●), from two or three experiments. At 0.4 μM ligand, when scattering ability was lowest and the initial volume increase was maximal, RBCs appeared identical to the stage 1 echinocytes created by DIDS. Increasing the azide to 3 μM generated highly spiculated, stage 3/4 echinocytes which exhibited efficient light scattering ability. Volume change was minor during this transition. Even higher azide doses caused progressive salt and water influx and cell swelling with eventual lysis and no steady 4 min signal could be obtained. Nearly identical effects were observed in K^+ PBS buffer [4]. At 60 μM, cells in *N*-methylglucamine buffer abruptly shrunk and scattering increased since K^+ efflux was no longer compensated by influx of the relatively impermeable cationic amine. Single values of 11.7 (×) and 13.1 (△) are scatter signals from cells 15 min (in Na^+ PBS) and 4 min (in K^+ PBS) after ligand addition. (Panel B) Coulter sizing measurements, showing an initial 10% increase in volume when 20–30 million azide molecules were added to each cell. The dose response tended to plateau at about 0.4–0.6 μM but was offset by a second volume increase occurring at slightly higher doses.

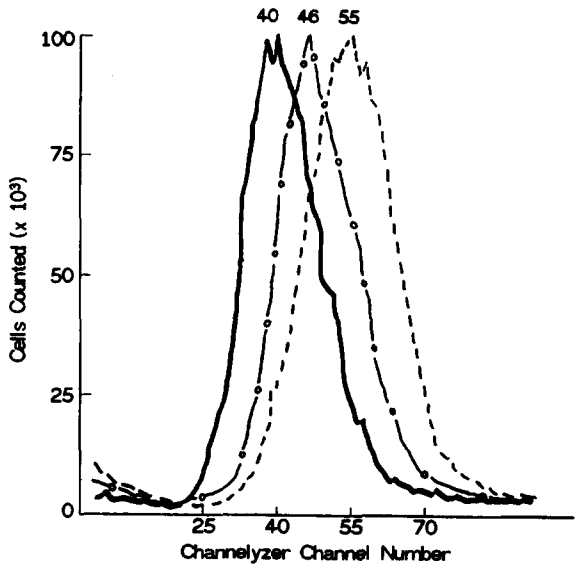

Fig. 4. Effect of photolysis on electronic sizing profiles of *p*-AzBPhz-treated cells. Channelyzer tracing (solid line; channel number 40, 89 fl) of control cell volume distribution. The dashed line with mean channel value of 55 (111 fl) was obtained when RBCs were exposed to 21 μM ligand and counted in Na^+PBS buffer containing the identical concentration of azide. If these treated cells were counted in azide-free buffer, ligand binding would reverse and the control curve would be obtained. Azide treatment followed by photolysis prevented the swollen cells' conversion to normal size during their assay in ligand-free buffer. Depending upon irradiation time, intermediate channel numbers were obtained; for example, 50 min photolysis produced cells that returned only partially to normal size (o——o), mean channel value of 46 (98 fl).

light decreased (Fig. 2A). The effect was maximal at 0.06 μM; thereafter, no further changes in cell structure or light scattering could be detected even when DIDS concentration was increased by three orders of magnitude.

Why do these cells scatter less light as they become studded with surface irregularities that should make them better deflectors? The results of concurrent Coulter Counter measurements provide an answer to this question (Fig. 2B). DIDS produced an 8–10% increase in MCV at the same dose that caused a maximal loss in light scattering ability. The agent forced the cells to attain a new steady state in which their hemoglobin is diluted and refractive index is decreased. No further DIDS-induced volume changes were observed until extreme concentrations were added (about 100 μM). This massive amount converted the cells to late stage echinocytes in which the blunt surface irregularities were transformed into elongated spikes (as in Fig. 4 IV, Ref. [4]). Cells in this form scattered more light.

3.3. Light scattering and cell volume alterations by p-AzBPhz

The scattering index and volume changes produced when red cells, in either Na^+PBS or *N*-methylglucamine buffer, were treated with *p*-AzBPhz over an extended concentration range are illustrated in Fig. 3. When 2–3 million molecules were added per cell, rapid cell flattening occurred (data not shown), the same as that produced by low dose DIDS. This was only occasionally detected as a small increase in light scatter. Addition of about $20 \cdot 10^6$ molecules azide/cell or 0.3–0.4 μM (equivalent to about 1 million molecules bound/cell; see Discussion) produced a maximal loss in light scatter as well as an 8–10% volume increase as detected by Coulter measurements. These DIDS-like, initial changes exerted by *p*-AzBPhz are hereafter referred to as the high-affinity, phase 1 effects. A plateau in the MCV dose-response curve was not quite attained because the addition of only slightly more azide produced a second membrane alteration, one involving a loss of integrity – one that culminated in cell lysis (the phase 2 azide effect). The azide-induced volume changes were observed in either Na^+PBS or K^+PBS buffers and even when the relatively membrane impermeable *N*-methylglucamine was the predominant cation (Fig. 3 A and B). No cation influx takes place during phase 1 and intracellular concentrations of Na^+ and K^+ actually decrease to the same extent as the RBC volume increase [4].

When the concentration of *p*-AzBPhz was increased by 2 log units greater than that required to produce maximal high-affinity effects, the cells sustained a second, non-specific alteration. During this phase 2 event, the light scattering pattern reversed twice. Cells first became more efficient light deflectors (scattering increased) as the blunt membrane projections progressively narrowed into more than 100 long spikes. Cell size remained nearly constant during this morphology change to stage 3 echinocytes. When the azide concentration reached about $2 \cdot 10^8$ molecules/RBC, the scattering signal abruptly reversed again. Cells swelled and became spherical. In sharp contrast to the phase 1 effects, this scatter signal did not stabilize within 1–2 min after ligand addition, reflecting the cell's inability to maintain a steady-state volume at these higher doses. The ligand apparently forms channels of a size large enough to permit the influx of salt and water, but not molecules the diameter of sucrose; at 25 mM, the impermeable sugar blocked the rate and extent of hemolysis at these high azide levels [5]. As long as *p*-AzBPhz remained less than 40 μM, the rate of salt and water influx was relatively slow and, as shown in Fig. 3A, swelling but very little lysis occurred within the 4 min measurement. Even after 15 min at 15 μM, only about 5% of the cells had lysed. However, at the

Table 1
Microhematocrit measurements on *p*-AzBPhz- and DIDS-treated RBCs

	Nominal μM [ligand]	Decrease in hemoglobin absorbance/mm of packet cells (%)	Trapped volume (%)
	0 (control)	(41)	5.8 ± 0.48 (28)
	0 (270 mosM)	9.7 ± 0.20 (7)	–
DIDS	0.002	1.6 ± 0.11 (14)	4.7 ± 0.37 (10)
	0.006	2.2 ± 0.20 (15)	4.6 ± 0.32 (12)
	0.01	3.3 ± 0.23 (19)	4.4 ± 0.26 (11)
	0.033	6.1 ± 0.47 (15)	4.3 ± 0.31 (12)
	0.10	8.0 ± 0.75 (13)	4.2 ± 0.24 (12)
p-AzBPhz	0.1	1.9 ± 0.36 (13)	4.7 ± 0.31 (12)
	0.2	3.2 ± 0.39 (17)	4.2 ± 0.41 (11)
	0.4	7.1 ± 0.45 (22)	4.1 ± 0.45 (14)
	1.0	7.4 ± 0.51 (15)	4.4 ± 0.54 (12)
	3.0	8.2 ± 0.80 (12)	7.8 ± 1.56 (12)

A counted number of RBCs in Na^+ PBS were treated with varying concentrations of *p*-AzBPhz and DIDS. Suspensions were spiked with ^{14}C-labeled inulin, loaded into calibrated microhematocrit capillaries and then centrifuged. After measuring the lengths of supernatant and cell column, the packed RBCs (range 16.0–20.3 mm) were extruded, lysed and assayed for radioactivity and hemoglobin. Hemoglobin/packed cell length and trapped fluid space both decreased depending on dose of either ligand, suggesting that cells packed tighter even though swollen. At 3 μM azide, the trapped volume increased reflecting the increased cell rounding that occurs during phase 2. The extent of swelling when RBCs were suspended in 270 mosM buffer (in lieu of normal 320 mosM) is shown for comparison. Compiled values represent means ± S.D. (*n*).

critical concentration of 60 μM, nearly complete lysis was seen in 30 min in Na^+PBS and within 4 min in K^+PBS. Presumably, lysis was slower in Na^+PBS because K^+ efflux compensated somewhat for the rapid Na^+ influx. However, when the predominant extracellular cation was *N*-methylglucamine, the opposite occurred – cell volume decreased and light scattering increased at 60 μM azide. Apparently, pores were created that were wide enough for the rapid escape of K^+ (plus Cl^- and water) but they still partially restricted the influx of the larger protonated amine.

3.4. Phase 1 swelling measured by microhematocrits

Table 1 displays the results of microhematocrit experiments performed to verify the phase 1 volume effects seen with the Coulter instrument. When erythrocytes were treated with varying amounts of *p*-AzBPhz or DIDS, the packed cell volume increased, the amount of hemoglobin/mm of packed cells was reduced, but less extracellular fluid was trapped in the packed cell column. These results indicate that the ligand-treated cells have a larger MCV and pack tighter, suggesting that they were more deformable, a finding consistent with our unpublished observations (see footnote 1 on p. 224).

3.5. Irreversibility of p-AzBPhz action after photolysis

As long as the ligand was tested below 1–2 μM in subdued light, its effects were completely reversed by simple dilution of the cell suspension with azide-free buffer. Fig. 4 replicates the Channelyzer tracings obtained for normal RBCs in Na^+PBS and cells treated with a large dose (21 μM) of *p*-AzBPhz. The mean channel value of 40 for control cells (89 fl; solid line) was shifted to 55 (111 fl) within 4 min after azide addition. However, a stable increase in size distribution could not be recorded unless the azide was also present in the Coulter counting buffer. If azide-treated cells were diluted 1: 500 in ligand-free buffer, the binding rapidly reversed and the Coulter signal reverted to the control profile during the 1–2 min measurement. However, if the membrane-bound ligand was first photolyzed at 255 nm and then diluted in the reservoir buffer, the return to normal MCV and shape upon dilution was incomplete. Cells exposed to UV for 20 or 70 min, and then diluted, generated profiles with channel values of 43 (nearly complete reversal) and 52 (almost no reversal), respectively. Photolysis for 50 min gave an intermediate value of 46 after dilution (Fig. 4). Relatively long periods of photoactivation were required because the cells were not stirred, the incident UV was of low intensity, and shielding by hemoglobin. RBCs irradiated for 70 min in the absence of azide produced a profile identical to that measured for non-irradiated controls. Which membrane components are photolabeled under these conditions has not yet been clearly established. In preliminary experiments, using tritiated *p*-AzBPhz as well as its aglycone ([1], and unpublished data), the only protein targets were Bands 3, 4.5 and particularly a 28 kDa protein [24].

3.6. Effects of DIDS and p-AzBPhz are additive

If *p*-AzBPhz's phase 1 effects are the result of its interaction with Band 3, then a decrement in this activity should be observed when the alleged common receptor site is occupied by DIDS. Ligands that act through the same mechanism will exhibit additive activity up to a common ceiling, and this was found for the stilbene and azide in a series of experiments (Fig. 5). Cells were first treated for 30 min with different concentrations of DIDS which exerted effects as those shown in Fig. 2. Equal aliquots were then placed into sample and reference cuvettes, and after the absorbance was re-balanced, varying concentrations of azide were added to only the experimental sample. Since DIDS had already exerted partial effects, the

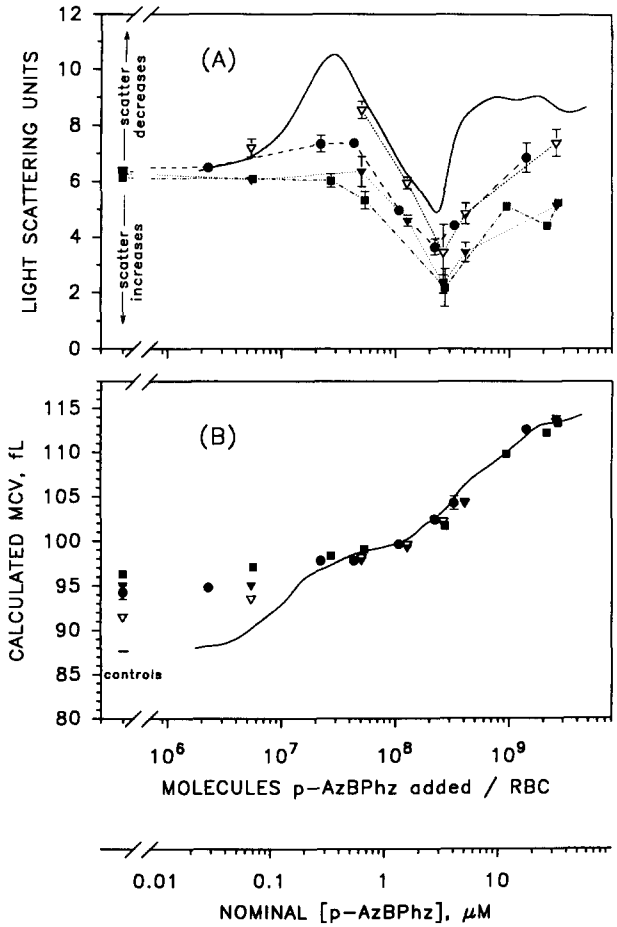

Fig. 5. Shape and volume changes of DIDS and *p*-AzBPhz are additive. RBCs were first treated with DIDS at 0 (solid line), 0.017 (▽), 0.2 (○), 1.7 (▼), and > 5 μM (■) for 30 min. Increasing DIDS progressively robs the azide of its capacity to elicit additional shape and volume changes normally produced at 0.06–0.6 μM (Panel A). However, cells exposed to even the highest dose of DIDS were still susceptible to *p*-AzBPhz's non-specific, phase 2 action but only after it attained the same concentration (0.6 μM) that would have caused these changes in non-DIDS treated cells. See text for further details. (Panel B) The solid line replicates the data in Fig. 3B. The volume of DIDS pre-swollen cells was relatively unaffected over the concentration range of azide normally producing phase 1 effects (up to 3 μM). DIDS treatment had no influence on the *p*-AzBPhz's ability to alter membrane integrity and increase cell volume at greater than 3 μM.

azide's ability to produce phase 1 action was limited to that residual from the maximal effect caused by either agent alone, viz. 4 scattering units and about 10 fl MCV increase. For example, *p*-AzBPhz could produce only about two additional scattering units in RBCs previously treated with 0.2 μM DIDS which had already influenced light scatter by 2 units (see Fig, 2). When phase 1 concentrations (0.06–0.6 μM) of the azide were added to cells that had already been maximally altered by DIDS, no further decrease in light scatter and only minimal MCV changes (from 97 to 99 fl) was detectable. On the other hand, DIDS-treated cells were fully responsive to the azide's phase 2 action. It is noteworthy that increased scatter due to the highly spiculated cell forms did not occur until the azide reached 0.6 μM, the same concentration required to alter untreated cells. The abrupt pre-lytic MCV increase and sphero-echinocyte formation was also initiated at 3 μM, in both untreated or DIDS-exposed cells.

4. Discussion

Two critical points need to be considered in assessing our results. The first pertains to our view that the Coulter Channelyzer signal is a reliable indicator of cell volume. Although the data in Fig. 1 support this idea, impedance measurements made with the Coulter instrument are known to be influenced not only by volume but also by the orientation and shape as well as the flexibility of the particle being measured. Skewed distribution curves and erroneously large RBC size estimations have been reported when MCHC values were high, cell deformability was reduced, or the orifice of the aperture tube was too small [25–27]. These factors are not applicable in our work; an optimal 100 μm dia orifice was used, the MCHC of ligand-treated cells was low, not high, and RBCs actually became more deformable when treated with low levels of azide (see footnote 1 on p. 224). Furthermore, there is at least one report that RBC shape changes introduced no significant error in volume determinations [28]. Finally, to rule out any ambiguity involving the Coulter signal, the phase 1 cell swelling was documented with microhematocrit measurements in calibrated capillary tubes ([4], and Table 1). Since < 1% hemolysis occurred, these data confirmed our earlier finding [4] indicating that treated cells packed tighter, despite being slightly swollen. This apparent increase in deformability was also found in unpublished experiments in which azide-treated RBCs (and especially sickle cells) passed through 3-μm pore filters more readily than controls.

The second point is that the phase 1 morphology and volume changes occurred when RBCs were exposed to only a few million ligand molecules/cell. At this low concentration, tritiated azide binding to 0.2% cell suspensions was found to be about 6–8% of that added (data not shown). Although binding was not determined at the 0.07% hematocrit used in the present study, we have extrapolated that about 2–3% of the added azide binds to the smaller number of cells. With this assumption, maximal phase 1 alterations occur when a cell binds about 1–2 million molecules, a value equivalent to the number of copies of Band 3. While this agreement may be fortuitous, the observation [1,3] that the anion transporter, operating in the

exchange mode, was about half-maximally inhibited at this azide concentration makes a strong argument for this relationship. Consequently, we propose that the first molecules of *p*-AzBPhz added to erythrocytes do not interact with the bulk lipid but are preferentially recruited by an external binding site on the transport proteins. Support for this idea comes from the cell sizing, microhematocrit and light scattering measurements showing that as long as the ligand was tested below 1–2 μM in subdued light, highly deformed and swollen cells rapidly reverted to normal sized discocytes after dilution in azide-free buffer. Our video imaging study indicated that this reversal takes place in seconds. Insertion into the bilayer occurs at higher dosages and this is when the non-specific phase 2 effects are observed. A similar bimodal interaction with the RBC membrane was reported for phloretin, from which *p*-AzBPhz is derived: Two sites were identified, one protein and the other lipid [29].

To our knowledge, the small increase in RBC volume caused by DIDS has been reported with minimal comment only twice before [8,30]. On the other hand, our findings do not agree with the work of Mosior and co-workers [31] who reported that DIDS did not alter cell volume of bovine RBCs washed in Mg^{2+}/Hepes buffer after DIDS treatment. The immense amount of DIDS required to initiate phase 2-like cell forms was also a surprising result. Much evidence [32–36] supports the view that the degree of echinocytosis induced by anionic amphiphiles is proportional to the amount inserted preferentially into the bilayer's outer leaflet. Anions more lipophilic than the stilbene would have produced DIDS-like membrane bulges as a first step in a continuum towards stage 3/4 echinocyte formation. In contrast, after DIDS produced its low dose morphology changes, stage 2 spicule formation did not take place over the next 1000-fold concentration increase. Consequently, it appears that identical initial shape changes can be produced by two different mechanisms; one the result of a Band 3 modification and the other consistent with the bilayer couple hypothesis [34].

Deuticke's early work [37] and the bilayer couple hypothesis [34] motivated several authors to suggest that normal discocytes convert to echinocytic forms when the outer leaflet selectively expands (or the inner contracts) by 0.4–1.2% [36,38,39]. In our work, the maximal increase in light scattering due to long spicule and stage 3/4 echinocyte formation required 3 μM azide. The expansion of the membrane surface area elicited by this high ligand level can be estimated by assuming that only the lipophilic benzylazide moiety inserts into the outer bilayer. This cylindrical moiety of about 0.5 nm dia could then occupy $0.2 \cdot 10^{-6}$ μm^2 and the $6 \cdot 10^6$ molecules bound to each cell (3% of that added) would occupy 1.2 μm^2. With the RBC surface area taken as 140 μm^2, the outer monolayer could have expanded about 0.8%. This calculation re-emphasizes the idea that the initial, phase 1 membrane deformations which require only 0.1–0.2 μM are not the result of this lipid insertion mechanism. Furthermore, it is generally accepted that anionic lipids act by an isovolumetric mechanism [36,40], but there are at least two contradictory reports [41,42]. Our results indicate that the initial morphology change produced by the Band 3 antagonists is accompanied by about a 10% water influx. This is not a dilemma; it indicates that water movement occurs only when the membrane distortion is Band 3 mediated and involves the weakening or remodeling of the dynamic interactions among the skeletal components [43].

How the interaction of our ligands with Band 3 could affect the cytoskeleton to cause the observed shape, volume and rheology changes remains unanswered. Considerable evidence supports the view that the functional integrity of the membrane skeleton and its linkage to the bilayer are essential for the maintenance of the red cell shape and mechanical soundness [17,18,44–46]. In one popular proposal, a translocase ('flipase') accounts for the asymmetrical assembly of phospholipids; acidic phospholipid asymmetry is maintained by their interaction with cytoskeletal components, primarily spectrin [17,47–52]. Cytoskeletal alterations causing withdrawal of the meshwork from the membrane's inner surface might then allow the inward-facing aminophospholipids to flop and expand the outer leaflet. This step-wise, spectrin withdrawal mechanism seems much too slow to account for the rapid initiation of stage 1 echinocytosis by our ligands.

Any proposal offered to explain the azide's phase 1 shape and volume effects must account for the following findings: (i) very rapid action and complete reversal within 1–2 s by simple dilution of the ligand/cell suspension (see footnote 1 on p. 224); (ii) stable cell alterations at each dose increment representing new steady state formation [5]; (iii) irreversibility of the effects after ligand photolysis (Fig. 3); (iv) activity in either Na^+, K^+ or *N*-methylglucamine Cl buffers and cell swelling independent of cation fluxes (Fig. 2 and Ref. [4]); (v) effects occurred when ligand binding was 10-fold less than the amount required to form echinocytes by outer membrane monolayer expansion; (vi) other agents possessing the benzylazide moiety (4-azidophlorizin, and the aglycone of *p*-AzBPhz) do not produce these effects at comparable doses (data not shown); (vii) the environment of a lipid specific spin label (5-doxylsteric acid) pre-inserted in the membrane was unaltered upon azide binding, whereas motion of a reporter group monitoring the lipid/water interface (Cat-16) became restricted [3]; (viii) a cell (membrane?) that becomes more deformable, not stiffer (see footnote 1 on p. 224); (ix) inhibition of the anion transporter over the same concentration range

[1]; (x) DIDS acted additively and produced the same phase 1 alterations as *p*-AzBPhz.

An explanation that satisfies each of these criteria and accounts for the morphological, rheological and volume alterations induced by either DIDS or the lowest levels of *p*-AzBPhz is that the ligands have a common mechanism, i.e., interaction with Band 3. Spicule formation, stage 3/4 echinocyte formation, loss in membrane integrity with eventual lysis results from the azide's interaction with membrane lipids.

It is still puzzling why water uptake should occur. What is the driving force for this influx? Vertessy and Steck [53] presented ideas which prompt us to consider a possible auxiliary role of the cytoskeleton, namely it provides the red cell membrane a means to resist stretching. Any relaxation of cytoskeletal infra-structure might allow, for a given osmotic force, a greater resting cell volume. Perhaps the Band 3 antagonists behave like Ca^{2+} which has been reported to induce localized detachment of skeletal proteins from the inner membrane face and produce the same cone-shaped membrane projections as seen with our ligands [54–56]. Could the drive for water influx be created by an alteration in the pre-existent intracellular osmotic solute concentration? A plausible mechanism might involve a ligand-induced exposure of new amine residues (perhaps from hemoglobin?) which could be protonated and provide the impetus for chloride entry [28]. However, a considerable number of moieties would have to be generated to foster a chloride influx satisfying the osmotic force requirements. In a preliminary experiment to monitor pH changes, a medium alkalization that should have resulted from proton influx was not detectable.

Acknowledgments

We are especially grateful to Richard Geissler for carrying out the scanning electron microscopy, and to Chris Maynard for his insight and fortitude during the initial stages of this study. This work was supported by a National Science Foundation Grant (RII-8610671) and the Commonwealth of Kentucky through the Kentucky EPSCoR Program.

References

[1] Diedrich, D.F. (1990) Methods Enzymol. 191, 755–780.
[2] Gibbs, E.M., Hosang, M., Reber, B.F.X., Semenza, G. and Diedrich, D.F. (1982) Biochim. Biophys. Acta 688, 547–556.
[3] Wyse, J.W., Blank, M.E., Maynard, C.L., Diedrich, D.F. and Butterfield, D.A. (1989) Biochim. Biophys. Acta 979, 127–131.
[4] Blank, M.E. and Diedrich, D.F. (1990) Biorheology 27, 345–355.
[5] Maynard, C.L. and Diedrich, D.F. (1988) Prog. Clin. Biol. Res. 258, 235–247.
[6] Forman, S.A., Verkman, A.S., Dix, J.A. and Solomon, A.K. (1982) Biochim. Biophys. Acta 689, 531–538.
[7] Frohlich, O. and Gunn, R.B. (1987) Am. J. Physiol. 252 (Cell Physiol. 21), C153–C162.
[8] Toon, M.R. and Solomon, A.K. (1987) J. Membr. Biol. 99, 157–164.
[9] Deuticke, B., Luetkemeier, P. and Poser, B. (1991) Biochim. Biophys. Acta 1067, 111–122.
[10] Andersen, O.S., Finkelstein, A., Katz, I. and Cass, A. (1976) J. Gen. Physiol. 67, 749–771.
[11] Cousin, J.L. and Motais, R. (1977) Biochim. Biophys. Acta 507, 531–538.
[12] Melnik, E., Latorre, R., Hall, J.E. and Tosteson, D.C. (1977) J. Gen. Physiol. 69, 243–257.
[13] Ehrenspeck, G. (1975) Experientia 31, 85–86.
[14] Jennings, M.L. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 381–397.
[15] Evans, J.O. and Diedrich, D.F. (1980) Arch. Biochim. Biophys. 199, 342–348.
[16] Singer, S.J. (1992) J. Membr. Biol. 129, 3–12.
[17] Middelkoop, E., Lubin, B.H., Bevers, E.M., Op den Kamp, J.A.F., Comfurius, P., Chiu, D.T.-Y., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1988) Biochim. Biophys. Acta 937, 281–288.
[18] Bennett, V. (1990) Physiol. Rev. 70, 1029–1065.
[19] Salhany, J.M., Cordes, K.A. and Gaines, E.D. (1980) Biochemistry 19, 1447–1454.
[20] Hsu, L. and Morrison, M. (1983) Arch. Biochem. Biophys. 227, 31–38.
[21] Adair, G.S. (1928) Proc. Royal Soc. London, Ser. A 120, 573–603.
[22] Williams, T.F., Fordham, C.C., III, Hollander, W., Jr. and Welt, L.G. (1959) J. Clin. Invest. 38, 1587–1598.
[23] Brecher, G. and Bessis, M. (1972) Blood 40, 333–344.
[24] Preston, G.M., Carroll, T.P., Gugguno, W.B. and Agre, P. (1992) Science 256, 385–387.
[25] Segel, G.B., Cokelet, G.R. and Lichtman, M.A. (1981) Blood 57, 894–899.
[26] Bator, J.M, Groves, M.R., Price, B.J. and Eckstein, E.C. (1984) Cytometry 5, 34–41.
[27] Waterman, C.S., Atkinson, E.E., Jr., Wilkins, B., Jr., Fischer, C.L. and Kimzey, S.L. (1975) Clin. Chem. 21, 1201–1211.
[28] Legge, D.G. and Shortman, K. (1968) Brit. J. Haematol. 14, 323–335.
[29] Owen, J.D. and Solomon, A.K. (1972) Biochim. Biophys. Acta 290, 414–418.
[30] Knauf, P.A., Fuhrmann, G.F., Rothstein, S. and Rothstein, A. (1977) J. Gen. Physiol. 69, 363–386.
[31] Mosier, M., Bialas, W.A. and Gomulkiewicz, J. (1988) Biochim. Biophys. Acta 945, 51–55.
[32] Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416.
[33] Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494–500.
[34] Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457–4461.
[35] Fugi, T., Sato, T., Tamura, A., Watkatsuki, M. and Kanaha, Y. (1979) Biochem. Pharmacol. 28, 613–620.
[36] Ferrell, J.E., Lee, K.-J. and Huestis, W.H. (1985) Biochemistry 24, 2849–2864.
[37] Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494–500.
[38] Beck, J.S. (1987) J. Theor. Biol. 75, 487–501.
[39] Lange, Y. and Slayton, J. (1982) J. Lipid Res. 23, 1121–1127.
[40] Lange, Y., Hadesman, R.A. and Steck, T.L. (1982) J. Cell Biol. 92, 714–721.
[41] Kwant, W.O. and Van Steveninck, J. (1968) Biochem. Pharmacol. 17, 2215–2223.
[42] Isomaa, B. and Paatero, G. (1981) Biochim. Biophys. Acta 647, 211–222.

[43] Edidin, M., Kuo, S.C. and Sheetz, M.P. (1991) Science 254, 1379–1382.
[44] Low, P.S., Willardson, B.M., Mohandas, N., Rossi, M. and Shohet, S. (1991) Blood 77, 1581–1586.
[45] Morrow, J., Cianci, C.D. and Giorgi, M. (1988) J. Cell Biochem. 37, 301–315.
[46] Reinhart, W.H., Sung, L.-P.A., Sung, K.-L.P., Bernstein, S.E. and Chien, S. (1988) Am. J. Hematol. 29, 195–200.
[47] Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
[48] Mohandas, N., Rossi, M., Bernstein, S., Ballas, S., Ravindranath, Y., Wyatt, J. and Mentzer, W. (1985) J. Biol. Chem. 260, 14264–14268.
[49] Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
[50] Frank, P.H.F., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, P.T.Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190.
[51] Schroit, A.J. and Zwaal, R.F.A. (1991) Biochim. Biophys. Acta 1071, 313–329.
[52] Comfurius, P., Bevers, E.M. and Zwaal, R.F.A. (1989) Biochim. Biophys. Acta 983, 212–216.
[53] Vertessy, B. and Steck, T.L. (1989) Biophys. J. 55, 255–262.
[54] White, J.G. (1974) Am. J. Physiol. 77, 507–514.
[55] Reinhart, W.H. and Chien, S. (1987) Am. J. Hematol. 24, 1–14.
[56] Henseleit, U., Plasa, G. and Haest, C. (1990) Biochim. Biophys. Acta 1029, 127–135.