Sulphydryl Reducing Agents and Shape Regulation in Human Erythrocytes

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Metabolic creation of red cells is reversible; on addition of nutrients, echinocytes recover the normal discoid shape. When the shape recovery takes place in the presence of reducing agents such as dithiothreitol (DTT), morphological change continues until the cells are stomatocytic. The degree of stomatocytosis varies, depending on the cell morphology when the nutrients and reducing agent are added. DTT has minimal effect on the shape of normal discocytes, but in its presence, mildly echinocytic cells become slightly cupped and advanced-stage echinocytes become severely stomatocytic. DTT must be present continuously for development and retention of stomatocytosis; echinocytes preincubated with or metabolically depleted in DTT do not become stomatocytic when supplemented in the absence of DTT, and DTT-induced stomatocytes revert to discocytes when the reducing agent is removed. DTT has no effect on adenosine triphosphate synthesis or equilibrium cell glutathione levels, and the induced stomatocytosis is not inhibited by excluding oxygen from cells during depletion. Spectrin phosphorylation and phosphate turnover are not affected by DTT. The echinocyte-to-discocyte transformation coincides with phosphorylation of membrane inner monolayer lipids (diacylglycerol to phosphatidic acid and phosphatidylinositol to phosphatidylinositol-4,5-bisphosphate). Overphosphorylation of these phospholipids is not responsible for the exaggerated shape recovery seen with reducing agents; phosphorylation of inner monolayer lipids proceeds identically in the presence and absence of DTT.

The normal discoid shape of the human erythrocyte can be altered in vitro by metabolic depletion, calcium loading, or addition of a variety of amphipathic agents. Control of normal cell shape has been attributed to the protein cytoskeleton that lamimates the cytoplasmic face of the membrane, with the suggestion that phosphorylation and dephosphorylation of spectrin could be the mechanism by which the cell regulates its morphology. While an intact cytoskeleton is necessary for shape changes to occur, recent work suggests that its role is passive in that it adopts the conformation imposed by the bilayer.

Another model for control of red cell shape is the bilayer couple hypothesis, which attributes cell shape changes to alterations in the relative areas of the inner and outer monolayers of the cell membrane. Any event or external agent that causes the outer monolayer to expand relative to the inner monolayer produces spiked spheres, echinocytes. Likewise, preferential expansion of the inner leaflet results in formation of cupped cells and spheres with invaginations (stomatocytes), species that appear to be the reverse of echinocytes. Agents that induce stomatocytosis can reverse the effects of echinocytic agents and vice versa.

Metabolic creation is thought to result from bilayer imbalance secondary to dephosphorylation of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid. This creation eventually becomes irreversible, but for many hours after adenosine triphosphate (ATP) depletion, addition of nutrients will induce echinocytes to revert to discocytes. The shape recovery coincides with phosphorylation of inner-membrane phospholipids that were dephosphorylated during creation.

Palek et al report that the reducing agent dithiothreitol accelerates the shape recovery of metabolically crenated cells, perhaps by reducing cross-linked cytoskeletal elements. In this paper, we report that DTT and other reducing agents effect further shape changes in intact cells, inducing them to form stomatocytes rather than reaching a stable discoid shape. The biochemical consequences of such reduction are examined.

MATERIALS AND METHODS

Blood was obtained from healthy adult volunteers by venipuncture and collected in EDTA, heparin, or citrate anticoagulant. Erythrocytes were separated from plasma by centrifugation at 1,800 g for five minutes and washed three times in 4 vol of 150 mmol/L NaCl. Cells were used within six hours of being drawn. If the erythrocytes were not used immediately after washing, they were suspended in 138 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L NaH₂PO₄, 6.1 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 5 mmol/L glucose, pH 7.4, and stored at 4 °C. Such cells were washed once in 150 mmol/L NaCl immediately before the experiment.

[³²]H₂PO₄ carrier-free) was purchased from ICN Radiochemicals (Irvine, Calif). Penicillin G was from Pfizer (New York). Luciferin, luciferase, 3,3'–dithiobis(6-nitrobenzoic acid) (DTNB), and DTT were obtained from Calbiochem–Behring (La Jolla, Calif). DTT was also purchased from Sigma (St Louis), which was the source of all other biochemicals. Other chemicals, obtained from J.T. Baker Chemical Co (Phillipsburg, NJ) or Fischer Scientific Co (Pittsburgh), were of at least reagent grade.

Film for autoradiography was obtained from Eastman Kodak Co (Rochester, NY); Cronex Lightning Plus intensifying screens were from DuPont Instruments (Wilmington, Del). Silica Gel HPL plates were purchased from Analtech Inc (Newark, Del).

Metabolic Depletion and Reversal

All incubations were carried out at 37 °C in capped plastic tubes. The incubation buffers contained 100 µg/mL penicillin and 100 µg/mL streptomycin to retard bacterial growth. To induce ATP depletion and crenation, cells were incubated in 4 vol of 150 mmol/L NaCl, 7.5 mmol/L Na₂HPO₄, pH 7.4, at 22°C (NaCl/P). Aerobic depletion was carried out in sealed tubes containing humidified N₂. The inert gas was bubbled through the cell suspension every ten hours or when tubes were opened for sampling, whichever was more frequent.

Shape recovery was effected by resuspending the pelleted echinocytes in 4 vol of 138 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L NaH₂PO₄, 6.1 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 5 mmol/L glucose, pH 7.4, and stored at 4°C. Such cells were washed once in 150 mmol/L NaCl immediately before the experiment.

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glucose, pH 7.4, supplemented with an additional 5 mmol/L glucose, 10 mmol/L inosine, and 1 mmol/L adenosine (supplemented NaCl/P). Stomatocyte formation was induced by adding DTT at a concentration of 10 mmol/L unless otherwise noted.

In some experiments, crenated cells were diluted to a hematocrit of 10% with NaCl/P and N-tosyl-L-phenylalanine chloromethyl ketone (TosPheCH2Cl, 200 mmol/L in dimethylsulfoxide) was added to yield a final concentration of 0.4 mmol/L. Control cells were treated similarly with dimethylsulfoxide alone.

In spectrin phosphorylation experiments, cells were labeled in 1 vol of 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mmol/L MgSO4, 1 mmol/L K2HPO4, 5 mmol/L KCl, 137 mmol/L NaCl plus 50 µCi/mL [32P]H3PO4 (HEPES/32P), supplemented with 10 mmol/L inosine, 10 mmol/L glucose, and 1 mmol/L adenosine. After ten hours the suspension was replenished with 10 mmol/L inosine, 10 mmol/L glucose, and 1 mmol/L adenosine. After 23 hours' incubation, by which time the amount of 32P in spectrin reaches a steady state,15 cells were pelleted, resuspended in HEPES/32P of the same specific activity, and allowed to deplete metabolically. Reversal of metabolic depletion was carried out in the same HEPES/32P, supplemented with sugars. Turnover and rephosphorylation of spectrin were examined in cells depleted in 32P-free NaCl/P, and resuspended in supplemented HEPES/32P.

In lipid phosphorylation experiments, cells were labeled with 32P by depleting them in 1 vol of NaCl/P, plus 150 µCi/mL [32P]H3PO4. After approximately 24 hours of incubation at 37°C, the cell suspension was diluted into 10 mmol/L Tris, 150 mmol/L NaCl (pH 7.4 at 22°C), 10 mmol/L glucose, 10 mmol/L inosine, and 1 mmol/L adenosine to give a final hematocrit of 20%.

**Assay Procedures**

**Morphology.** Erythrocytes were diluted to a hematocrit of 5% and fixed in 150 mmol/L NaCl containing 0.5% glutaraldehyde. Cells were examined by bright field microscopy at a magnification of x 500. Stomatocytes were assigned a score of −4 to −1,18 discocytes a score of 0, and echinocytes a score of +1 to +5.18 The average score for a field of 100 cells was called its morphological index (MI).17 Some fixed cell samples were washed with distilled water, placed on glass coverslips, air-dried, and examined by scanning electron microscopy.

**Adenosine triphosphate.** Frozen aliquots of cell suspension (50 µL) were diluted in 2.45 mL 150 mmol/L NaCl, boiled for 15 minutes, and centrifuged to clarity. The supernatant (undiluted or diluted 1:10) was assayed for ATP by the luciferin-luciferase method.19

**Reduced glutathione.** Reduced glutathione was measured by the DTNB reagent method20 on frozen acid lysate of cells, unless otherwise noted. When present, DTT was removed from cell suspensions by repeated washing to remove the DTT, which also reacts with DTNB.

**Spectrin phosphorylation.** Aliquots (150 µL) of 20% cell suspension were lysed in 1.2 mL 10 mmol/L Tris, 2 mmol/L EDTA, pH 7.5, at 22°C immediately after removal from incubation. The stroma were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% slab gels.20 Autoradiograms of the dried gels were obtained by one-day exposures to Kodak X-OMAT AR film with intensifying screens. Individual bands of the gels were cut out, dissolved in 1 mL 30% H2O2 at 60°C for nine to 24 hours, and analyzed by liquid scintillation counting. Results were normalized to the amount of protein loaded onto the gel, as determined by the Lowry assay.21

**Lipids.** Aliquots (100 µL) of cell suspension were removed during the shape recovery and stored frozen. Phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate, and phosphatic acid levels were measured as described previously.22 The frozen aliquots were lysed in 2 mL cold 10 mmol/L Tris, 2 mmol/L EDTA (pH 7.5 at 22°C) and washed once in the same buffer. The pellet was extracted with 1.8 mL methanol/chloroform/concentrated HCl, 100:50:1 (vol:vol:vol), followed by water (0.6 mL) and chloroform (0.6 mL). Aliquots (0.7 mL) from the organic phase were dried under a stream of N2 and redissolved in three 5-µL portions of chloroform. Lipids were separated by thin-layer chromatography on nonactivated 2.5 x 10-cm plates in 48:40:10.5 (vol:vol) methanol/chloroform/H2O/ concentrated NH4. Lipid spots were detected by iodine vapor and autoradiography. 32P-labeled phospholipids were quantified by liquid scintillation counting of scraped spots.

**RESULTS**

Erythrocytes were incubated in NaCl/P, to effect metabolic depletion. As shown previously,1,12 ATP depletion proceeded with a half-time of five to ten hours and was followed by metabolic crenation. Metabolically depleted echinocytes (Fig 1A) were pelleted and resuspended in sugar supplemented NaCl/P, ATP levels were partially restored and the cells recovered normal discoid shape, remaining in this configuration for many hours as long as nutrients were available (Fig 1B and Fig 2). DTT altered this shape recovery dramatically. In the presence of 10 mmol/L DTT, echinocytes quickly recovered discoid shape and then became cupped and invaginated stomatocytes (Fig 1C and Fig 2). The extent of the morphological overshoot depended on the original extent of crenation. Stage 1 echinocytes attained a slightly cupped shape, while severely crenated cells eventually developed the membrane invaginations of advanced-stage stomatocytes (Fig 3A). Similarly, if echinocytes had partially recovered toward discoid shape before addition of the reducing agent, the extent of DTT-induced stomatocytosis diminished (Fig 3B).

Other sulfhydryl reducing agents also induced stomatocytosis in recovering echinocytes but to variable extents. Dithioerythritol produced effects similar to the DTT response and in the same concentration range. 2-Mercaptoethanol induced detectable but less severe stomatocytosis; even at concentrations greater than 50 mmol/L this reagent typically produced morphological indices no greater than −1.0 (data not shown). Echinocytes treated with sodium dithionite (Na2S2O4, 10 mM) did not recover discoid or stomatocytic morphologies in the presence of sugars; thus, some reducing agents inhibit even normal shape recovery.

The continuous presence of the reducing agent was required to generate and maintain stomatocytic morphologies. Echinocytes pretreated with DTT (10 mmol/L for two hours at 0°C) and then washed free of DTT before nutrient supplementation recovered the normal discoid shape but did not cup (Fig 4A). Identical results were obtained with DTT pretreatment at 37°C. When DTT-induced stomatocytes were washed free of the reducing agent, the cells slowly recovered discoid shape (Fig 4B).

The extent of induced stomatocytosis was dependent on the DTT concentration. At hematocrits of 5% and 20%, half-maximal stomatocytosis was obtained at DTT concentrations of 2 to 3 mmol/L (Fig 5A). The same concentration dependence was observed for cells beginning at different
Fig 2. Effect of DTT on the shape recovery of crenated cells. Erythrocytes were metabolically depleted for 24 hours and then resuspended in supplemented NaCl/P, (O) or in supplemented NaCl/P, plus 10 mmol/L DTT (●).

Oxidative damage. To examine the role of oxidative damage in DTT-induced stomatocytosis, cells were depleted under an inert atmosphere and in air. The inert atmosphere slowed glutathione depletion (Fig 6) but did not alter the rate of crenation. On sugar supplementation, erythrocytes depleted under an inert atmosphere recovered the normal discoid shape more rapidly than cells depleted under air, as reported previously.14 When supplemented in the presence of DTT, cells depleted under both conditions formed stomatocytes at the same rate and to the same extent (Fig 7).
The continuous presence of DTT is required for stomatocytosis. (A) Cells were metabolically depleted for 24 hours. Cells were treated with 10 mmol/L DTT at 0 °C for two hours and washed repeatedly (□, ▽) or not treated (○, ●). Cells were then resuspended in supplemented NaCl/P, without DTT (○, △) or with 10 mmol/L DTT (△, ▽). (B) Erythrocytes were depleted for 23 hours and resuspended in supplemented NaCl/P, without DTT (○) or with 10 mmol/L DTT (△). At t = 0.5, 1, 2, and 4.26 hours after resuspension in the DTT-containing buffer, aliquots were removed, washed extensively, and reincubated in DTT-free supplemented NaCl/P, (○).

Similarly, cells depleted in 2 mmol/L DTT and then washed to remove the reducing agent recovered discoid shape. In the presence of additional DTT, they formed stomiatocytes at the same rate and to the same extent as control cells (data not shown).

N-tosyl-L-phenylalanine chloromethyl ketone experiments. The chloromethyl ketone, TosPheCH2Cl accelerates the shape recovery of echinocytes obtained by incubation with dinitrophenol. TosPheCH2Cl did not alter the shape recovery of metabolically crenated cells, but in its presence, DTT induced greater than normal stomatocytosis (Fig 8).

ATP and glutathione. Glutathione and ATP levels were assayed during shape recovery. ATP levels typically recovered to about 40% of their normal value, both in control and DTT-treated cells (Fig 9). Glutathione levels recovered to approximately 50% of the value found in fresh cells. DTT reduced all available oxidized glutathione within 20 minutes (the minimum time to prepare samples for the glutathione assay), but it had no effect on the final concentrations of reduced glutathione (Fig 9).

Spectrin phosphorylation. Spectrin, the major cytoskeletal protein, is phosphorylated in the band 2 subunit. The level of phosphorylation decreases after metabolic depletion and crenation and increases again when sugars are added. Spectrin phosphorylation was not altered in echinocytes recovering in the presence of DTT. Cells prelabeled with 32P were metabolically depleted and then resuspended in sugar.
supplemented buffer containing $^{32}$P, at the same specific activity as the buffer used for labeling and depleting the cells. DTT did not affect the rate or extent of spectrin phosphorylation (Fig 10A); thus, spectrin phosphate levels were not altered by the reducing agent. To determine whether spectrin phosphate turnover is linked to DTT-induced stomatocytosis, cells were metabolically depleted in $^{32}$P-free buffer. The resulting echinocytes were resuspended in sugar-supplemented buffer containing $^{32}$P. The ensuing incorporation of $^{32}$P into spectrin should be due both to spectrin rephosphorylation and to turnover of residual phosphate groups. DTT had no effect on phosphate incorporation under these conditions (Fig 10B); thus, it does not alter the rate of turnover of spectrin phosphate.

Phosphoinositide and diacylglycerol phosphorylation. Metabolic crenation of erythrocytes coincides with the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and of phosphatidic acid to diacylglycerol. Lipid dephosphorylation is reversed by the addition of sugars and accompanies the shape recovery.12 The phosphorylation of these lipids was examined in DTT-induced stomatocytes. Cells were metabolically depleted in the presence of $^{32}$P. The resulting echinocytes were incubated with sugars in the presence and absence of DTT. The shape recovery to discocyte and the DTT-induced stomatocyte formation were both accompanied by an increase in the $^{32}$P incorporated into phosphatidylinositol-4,5-bisphosphate, a decrease in phosphatidylinositol-4-phosphate levels and no change in the levels of phosphatidic acid (Fig 11). DTT had no effect on the amount of $^{32}$P incorporated into phosphatidylinositol-4,5-bisphosphate, phosphatidylinositol-4-phosphate, phosphatidic acid, or any other phospholipid.

**DISCUSSION**

The sulfhydryl reducing agent DTT has no evident effect on the morphology of normal human discocytes or echinocytes generated by metabolic depletion. However, when DTT is present during ATP repletion, echinocytes recover, not to discocytes, but to stomatocytes. The severity of the stomatocytosis is a function of the degree of initial crenation; more severely echinocytic cells cup more extensively. This is true both for cells depleted for varying lengths of time and for partially recovered echinocytes incubated in supplemented medium for varying lengths of time. Thus, DTT intervenes in some way in an ATP-dependent mechanism that restores and regulates cell shape.

**Oxidative damage of cell components.** The stability of ghosts and of cytoskeletal protein complexes depends on the integrity of sulfhydryl groups in actin and band 3.23 Palek et al14 found that DTT accelerates sugar-dependent shape recovery of metabolically depleted echinocytes.* They proposed that the cytoskeleton undergoes oxidative damage during depletion and that such damage must be repaired during repletion before the cell can assume its normal shape. These considerations raised the possibility that DTT affects cell shape by reducing certain protein sulfhydryl groups.

*These authors do not report finding stomatocytes in the population of recovered cells. The discrepancy between their results and the present findings may be due to differences in experimental conditions or in the method of assigning cell morphology.
DTT-INDUCED STOMATOCYTOSIS

Fig 10. Spectrin phosphorylation and DTT-induced stomatocytosis. (A) Net phosphorylation in the band 2 region. Cells were pre-labeled with $^{32}$P, metabolically depleted for 32 hours, pelleted and resuspended in sugar-supplemented $^{32}$P-containing buffer with (O) or without 10 mmol/L DTT (O); $t_{1/2}$ of shape recovery = 1.5 hours. Values are the average of three measurements. (B) Rephosphorylation and turnover. Cells were depleted for 23 hours, pelleted, and resuspended in sugar-supplemented $^{32}$P-containing buffer with (M) or without 10 mmol/L DTT (O); $t_{1/2}$ of shape recovery = two hours. Values are the average of four measurements.

Fig 11. Phosphoinositide metabolism and DTT-induced stomatocytosis. Cells were metabolically depleted in $^{32}$P-containing buffer for 21 hours and then diluted in sugar-supplemented buffer with or without 10 mmol/L DTT; $t_{1/2}$ of shape recovery = two hours. Phosphatidylinositol bisphosphate (O, O), phosphatidylinositol monophosphate (O, M), and phosphatidic acid (A, A). No differences in any lipid concentration were observed in the absence (O, O, A) or presence (O, M, A) of 10 mmol/L DTT. Values are the average of four measurements.

Beyond the extent normally attained in metabolic processes, releasing oxidative constraints to an abnormal degree. If the susceptibility of these proteins to such alteration were increased by the putative oxidative damage, the DTT effect might manifest itself, as observed, only in cells recovering from crenation.

This possibility was examined in experiments in which cells were allowed to crenate in the absence of oxygen or in the continuous presence of DTT. Neither of these treatments affects the degree of stomatocytosis observed when the echinocytes were supplemented with sugars and DTT. Alternatively, echinocytes generated under the usual experimental conditions were incubated for two hours with DTT and then supplemented with nutrients and allowed to change shape in the absence of DTT. These cells recover discocytic shape but do not become stomatocytic. Thus, protection of cells from oxidative damage does not abolish the effects of DTT.

Time course and concentration dependence of DTT responses. DTT must be present continuously not only for stomatocyte formation, but also for maintenance of the stomatocytic shape. When the reducing agent is removed, stomatocytes slowly revert to discocytes. This "relaxation" occurs on a longer time scale than the initial stomatocyte formation, indicating that the cell is slow to overcome the effects of DTT. The relaxation observed might be a reversal of the stomatocytic process, occurring at a slower rate because the cell's metabolic activity has reached a steady state; or it might involve a separate mechanism. It is perhaps significant that the time scale of stomatocyte reversal approaches the time scale of phospholipid flip-flop.

DTT does not act in a stoichiometric fashion. Half-maximal stomatocytosis is observed at 2 to 3 mmol/L DTT, regardless of the initial degree of crenation or the cell concentration. This finding, together with the requirement for continuous presence of DTT, is more consistent with modulation of an enzyme activity than with direct reductive action on cytoskeletal protein structure.

Other reducing agents. Sulphydryl reducing agents other than DTT induce stomatocyte formation in metabolically depleted echinocytes. Dithioerythritol effects stomatocytosis at the same concentration and to the same extent as does DTT. 2-Mercaptoethanol also induces stomatocyte formation but to a lesser extent, even at tenfold higher concentrations. The relative effects of mercaptoethanol and the bivalent reagents may reflect differences in their reduction potentials. The anomalous electrode behavior of thiols in aqueous solutions has made reliable determination of their relevant electrode potentials difficult, but relative values for mercaptoethanol and DTT may be inferred from their equilibrium constants for reduction of protein sulphydryl groups: the value for mercaptoethanol is approximately unity, $10^4$ less than that for DTT and dithioerythritol.

Metabolic effects. ATP and glutathione are metabolic intermediates depleted during crenation and regenerated as cells return to discocytes. Neither process appears to be the target of DTT. The rate and extent of ATP resynthesis are not affected by DTT. Cells depleted under nitrogen retain higher levels of reduced glutathione than cells depleted in air, but DTT induces stomatocytosis in cells of both types at the
same rate and to the same extent. DTT reduces oxidized glutathione within the first 20 minutes of incubation, but normal metabolic processes restore control cells to the same level well before stomatocytosis develops, and DTT does not alter the final level of reduced glutathione in the cell.

**Protein phosphorylation.** The rephosphorylation of spectrin, once thought to be the event affecting the echinocyte-to-discocyte transformation, is not altered during the echinocyte-to-stomatocyte transformation. DTT does not change the rate of spectrin rephosphorylation, nor does it alter phosphate turnover in band 2. No differences in phosphorylation of other membrane proteins are observed in DTT-treated and control cells.

**Phosphoinositide metabolism and bilayer balance.** The discocyte-to-echinocyte transformation in red cells is coupled to the metabolism of cell phosphoinositides. During metabolic depletion, crenation coincides with the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and phosphatic acid to diacylglycerol. The reversal of the shape change by the addition of nutrients reverses this lipid dephosphorylation. Phosphoinositide phosphorylation is not responsible for DTT stomatocytosis; the rate of formation and equilibrium levels of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid are indistinguishable in recovered discocytes and in DTT-induced stomocytes. If the apparent connection between phosphoinositide phosphorylation and cell shape is due simply to steric effects on membrane bilayer balance, as suggested earlier, the present findings would indicate that more than one metabolic mechanism influences cell shape. The morphology change from echinocyte to discocyte to stomocyte has been viewed as a continuum, as in the case of amphiophathic intercalation. If metabolically mandated shape changes are also governed by a single biochemical mechanism, it is more complex than simple phosphoinositide occupancy of inner monolayer area.

From presently available evidence, DTT-induced stomatocytosis could be a bilayer effect, a cytoskeletal effect, or both. The time scale of the process is consistent with the rate of phosphatidylerine translocation across the bilayer. Possibly, DTT activates a phospholipid translocation protein, causing net transfer of phospholipid to the inner monolayer. Alternatively, DTT may directly or indirectly alter cytoskeletal protein interactions, either among themselves or with the bilayer by way of the formation of a metabolite such as a polyanion. Our preliminary results may support the last hypothesis. Conditions that elevate cell 2,3-diphosphoglycerate levels during shape recovery induce additional morphological anomalies, which are under further investigation.

Cells incubated with the amphiophath dinitrophenol crenate, presumably due to the intercalation of dinitrophenol into the cell outer monolayer. The echinocytes generated by this agent revert slowly to discocytes in the presence of glucose. This shape recovery is accelerated by TosPheCH2Cl and hexose monophosphate shunt activators in normal cells, an effect not found in glucose-6-phosphate-dehydrogenase-deficient cells. The shape recovery from dinitrophenol crenation may be related to DTT-induced stomatocytosis. TosPheCH2Cl has no effect on the shape reversion of echinocytes to discocytes, but it potentiates the stomatocytic effect of DTT. This provides additional evidence that enzymes of the metabolic cycles are targets for the reducing agents.

**Morphology control in ghosts.** Under some conditions, ghosts resealed around physiological concentrations of MgATP form echinocytes that gradually become discoid and then stomatocytic. These shape changes coincide with formation of phosphatidic acid, and phosphatidylinositol-4,5-bisphosphate may also be elevated under some conditions. The degree of stomatocytosis eventually attained in ghosts depends on the degree of washing they receive as well as the buffer and temperature used: greater stomatocytosis and endocytosis are observed with ghosts washed extensively before resealing (unpublished observations, 1984). It is possible that ghosts and DTT-treated echinocytes share the same biochemical lesion that prevents them from maintaining discoid shape.

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Sulfhydryl reducing agents and shape regulation in human erythrocytes

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