# Deformation of transforming red cells in various pH solutions

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*Summary.* Centrifugation into a rapid cell fixative is used to determine the conformation of red cells at various pH-values as a measure of red cell deformability. Discocytes show a good deformation into handbag-like forms; echinocytes and stomatocytes have reduced deformability with characteristic shapes of deformation.

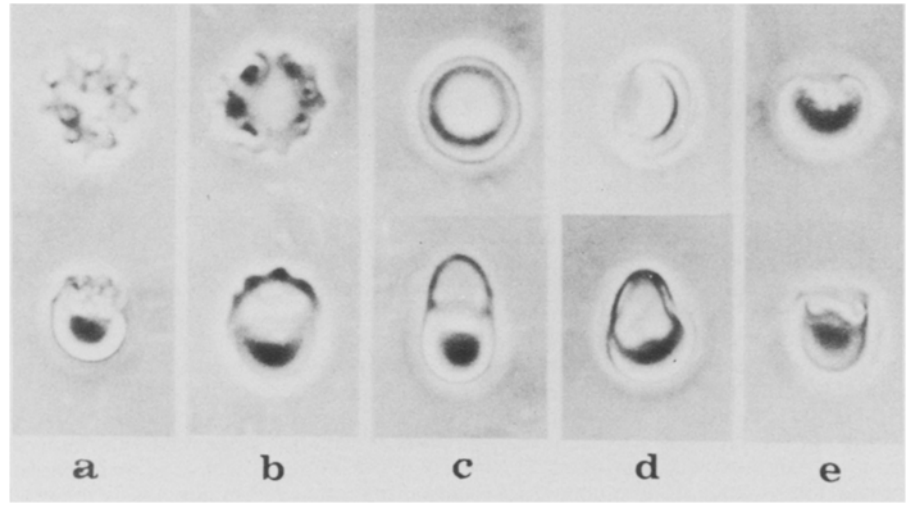
Red cell deformation occurs during passage through capillaries, and red cells with reduced deformability are removed from the circulation by the reticuloendothelial system. Measurements of red cell deformability are useful for the investigation of the viscoelastic properties of red blood cell membranes.

Red cell deformability is measured by viscometry[1], the micropipette technique[2], filtration[3], resistive pulse spectroscopy[4], ektacytometry[5] and centrifugal elongation[6-8]. Echinocyte and stomatocyte transformations occur at elevated and low pH in vitro, respectively. The application of a capillary tube centrifugal technique to evaluate changes in red cell deformability in centrifugal fields at various pH-values is reported.

*Material and methods.* To observe the deformation of human red cells, 5 µl of whole blood was suspended in 5 ml of Eagle MEM medium, and 50 µl of this suspension was filled into the top fraction (1.5 cm) of the siliconized capillary tube (8 cm length, 2 mm inner diameter). The middle fraction (5 cm) of the tube contained isotonic phosphate buffer solution prepared by the method of Clark and Shohet[9]. In the bottom fraction (1.5 cm) the diluted phosphate buffer solution (180 mosmoles) containing 1% glutaraldehyde (total osmolarity; 290-295 mosmoles) was present to fix the deformed cells. The capillary tube was quickly spun at 12,000 rpm for 1 min at 37 °C and the red cells were subjected to the centrifugal forces which induced cell deformation. The resulting deformation was evaluated by measuring the elongation of the red cells using a phase contrast microscope.

*Results and discussion.* In this technique the centrifuge had quickly accelerated to the desired high speed (12,000 rpm) by the time the red cells of the top fraction reached fixative at the bottom. The fixative completely fixed the deformed cells and no cell fragments were seen, suggesting that no mechanical hemolysis had taken place. The pH of the phosphate buffer solution of the middle and bottom fractions was varied by mixing various ratios of monobasic and dibasic stock solutions, and red cell deformability was measured at pH 8.5, 8.0, 7.4, 6.0 and 5.2. Red cells from the top fraction of the capillary tube transformed and deformed in the solution in the middle fraction and were fixed in the bottom fixative. The top row of the figure shows the discocyte-echinocyte-stomatocyte transformation as a function of pH and the bottom row presents the red cell deformation induced by centrifugal forces in the capillary tube. At pH 7.4 cell shape is a biconcave disc and centrifugal forces deform it to a handbag-like form. This deformation can be readily understood from consideration of the stresses acting on a cell. In the handbag shape, the flattened end portion of the cell is squeezed so it is empty of cell contents. The degree of displacement of the membrane depends on the cell deformability and the axis length of the elongated cell was $9.9 \pm 0.2$ µm. Increased pH transforms discocytes to echinocytes. The deformed cell at pH 8.0 has compressed spicules around the flattened end portion of the cell like an engagement ring form, and its elongation was $9.6 \pm 0.2$ µm. The echinocyte III, transformed in phosphate buffer solution of pH 8.5 (the buffering capacity at this pH is not so high) has reduced elongation ($7.8 \pm 0.4$ µm) and an octopus-like form, as in the figure; this suggests that poorly deformable spicules occupy the cell membrane and increase the membrane stiffness. At decreasing pH discocytes change rapidly to stomatocytes and spherostomatocytes. The deformed cell at pH 6.0 is a shellfish-like form which has a bulge and a tail portion with cell contents. Its elongation was $8.3 \pm 0.2$ µm. The commenest shape of the deformed cell at pH 5.2 is a jellyfish-like form which has poor deformability (cell elongation; $7.5 \pm 0.5$ µm).

Cellular deformability using this technique was evaluated by measuring the increase in cellular dimensions under applied centrifugal forces. The advantage of the present technique is that the pH effect on the cell can be detected during the cell deformation process in the capillary tube. Echinocyte formation at alkaline pH is overcome at the




Typical shapes at no stress (top row) and at an applied centrifugal force of 12,000 rpm (bottom row) of red cells at pH 8.5 (a), 8.0 (b), 7.4 (c), 6.0 (d) and 5.2 (e).

leading edge of descending red cells. Stomatocyte predilection at acid pH is modified as a thickening of the advancing edge and a thinning of the trailing edge. Red cell deformability as measured by ektacytometry is controlled by the relationship of the surface area to the volume of the cell, the internal viscosity of hemoglobin and the intrinsic rigidity of the red cell membrane[10]. In this technique, using a phosphate buffer solution at pH 7.4, factors that determine the elongation of the trailing edge include the relationship of surface area to volume and intrinsic characteristics of the membrane itself, because the elongated end portion of the cell becomes squeezed free of cell contents. The photographs suggest that it is more likely that the echinocytes and possibly even the stomatocytes have a smaller surface area than the normal red cells, and it is of interest that the echinocyte can lose some of its spicules when force is exerted on the inside of the membrane. This simple and inexpensive technique using a capillary tube will be useful for the investigation of rheological membrane properties.

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## Increased urinary excretion of cyclic nucleotides in X-linked hypophosphatemic *(Hyp)* mice

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*Summary. Hyp* mice, a model for human X-linked hypophosphatemia, had elevated urinary cyclic AMP, cyclic GMP, and magnesium excretion compared to normal mice. The data suggest a renal origin of the urinary cyclic nucleotides. No significant differences in plasma cyclic AMP and cyclic GMP were observed between genotypes.

*Introduction.* A mutant, X-linked dominant gene *(Hyp)* has been reported in mice[2] which results in a phenotype similar to that in human X-linked hypophosphatemia (XLH), a form of vitamin D-resistant rickets[3]. The disease in mice, as in humans, is characterized by increased renal excretion of phosphate (P), low plasma P (hypophosphatemia) and osteomalacia[2-4]. The etiology of XLH is unknown. Elevated urinary cyclic AMP (UcAMP) has recently been reported in *Hyp* mice[5-7]. But in those studies plasma cAMP was not measured, and consequently, it was not possible to determine whether the high UcAMP was simply due to elevated filtered load. Our study was undertaken to determine if the elevated UcAMP in *Hyp* mice was of renal origin. In addition, urinary excretion of cyclic GMP (UcGMP) - a parameter previously unexamined in XLH - was measured because cGMP, like cAMP[8,9], appears to be involved in the regulation of calcium and phosphate[10,11]. Finally, renal excretion of electrolytes was measured in order to confirm that the *Hyp* mice used in these studies had elevated excretion of P, and to determine if the renal excretion of any other electrolyte was also elevated.

*Materials and methods.* The breeding and maintenance of normal and *Hyp* C57BL/6J mice has previously been described[4]. The mean age of intact normal and *Hyp* mice used in this study was $13.5 \pm 0.1$ weeks, mean $\pm$ SE. Immediately prior to blood collection mice were held over a beaker and the spontaneously voided bladder urine was collected. Blood was then collected into heparinized microfuge tubes by cutting the carotid artery. Cyclic nucleotides were measured in urine and plasma samples by the double antibody radioimmunoassay (RIA) developed according to the method of Steiner et al.[12] using reagents commercially available from New England Nuclear (Boston, Mass; RIA kits NEX-132 and NEX-133). The validity of the RIA was verified in our laboratory by standard procedures[13]. Treatment of samples with phosphodiesterase (PDE) demonstrated the absence of immunoreactive substances (other than cyclic nucleotides) in plasma and urine. We have found that plasma PDE activity causes degradation of plasma cyclic nucleotides resulting in low plasma cyclic nucleotide values[14,15]. This problem can be minimized by rapid processing of samples - in our laboratory PDE was inactivated within 5 min after the onset of blood drawing by adding plasma to TCA. Based on degradation curves determined in our laboratory (not shown), the data shown herein represent at least 90% of the actual plasma cyclic nucleotide levels. Ether anesthesia did not influence plasma cyclic nucleotide levels. Procedures for measuring electrolytes have previously been described[4]. Plasma and urinary creatinine were analyzed using a modification of the kinetic method of Lustgarten and Wenk[16]. The fractional excretion (clearance ratio) of cyclic nucleotides was calculated as follows: fractional excretion (FE) = (urine/plasma concentration of cyclic nucleotide) $\div$ (urine/plasma concentration of creatinine) $\times$ 100.

*Results.* No significant difference (NSD) in plasma cyclic nucleotides was seen between *Hyp* and normal mice. Compared to normal miee, *Hyp* mice displayed significantly elevated UcAMP and UcGMP regardless of whether data were expressed as a fractional excretion or as nmole cyclic nucleotide/mg urinary creatinine. No significant genotype differences were observed for renal excretion of Ca (table) or Na and K (data not shown). The high FE-P, characteristic of XLH, was expressed to the same degree in both heterozygous *Hyp* females and hemizygous *Hyp* males ($40 \pm 4\%$ in *Hyp* females, $n = 14$, vs $21 \pm 2\%$ in normal females, $n = 17$; $37 \pm 4\%$ in *Hyp* males, $n = 16$, vs $21 \pm 3\%$ in normal males, $n = 17$, $p < 0.001$). Excretion of Mg was significantly greater in pooled *Hyp* mice compared to normals, confirming our preliminary report[15]. Elevated urinary Mg excretion in *Hyp* mice has also been observed by others[7], and may be related to the altered Mg metabolism previously reported[4].