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VITAMIN B_6 DEFICIENCY AND GALACTOSE INDUCED ALTERATIONS IN MORPHOLOGY AND OSMOTIC FRAGILITY OF RAT ERYTHROCYTES

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Abstract

Recently, red blood cells have been investigated mainly for alterations in ion transporting capacity, membrane bound enzymes or modifications in the structure of its individual constituents in clinical and experimental urolithiasis. However, the implication of such modifications on the physical state or morphology of cells has not been investigated. Scanning electron microscopic studies performed in vitamin B_6 deficient and/or galactose fed rat (established hyperoxaluric models) erythrocytes, showed the presence of large number of stomatocytes, spherocytes and other variously deformed cells as compared to discocytic cells seen in normal control group. These changes in shape were in concurrence with red cell osmotic fragility, which decreased both in vitamin B_6 deficient and vitamin B_6 deficient + galactose fed group (19% and 33% hemolysis at 4 g/l NaCl, respectively) while it increased in galactose control group (73% hemolysis at 4 g/l NaCl) as compared to normal control group (55% hemolysis at 4 g/l NaCl). These morphological and physical state alterations could be correlated with red blood cells' membrane cholesterol and phospholipid sub-class distribution. These findings suggest that some structural membrane changes occur due to vitamin B_6 deficiency and/or galactose feeding, which may be responsible for the altered membrane functions known to be associated with pathogenesis of urolithiasis.

Key Words: Scanning electron microscopy, erythrocyte shape, osmotic fragility, vitamin B_6 deficiency, galactose, hyperoxaluria, membrane lipids.

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Introduction

Extensive research in the field of membrane biochemistry in the last decade has attributed a number of metabolic defects, inherited or acquired, to the altered state of membrane. Several lines of evidence, for example, increased oxalate transport in intestine and kidney (Gupta *et al.*, 1988), enhanced oxalate binding to inner mitochondrial membrane and intestinal brush border membrane (Laxmanan *et al.*, 1986; Koul *et al.*, 1991), crystal induced membranolysis (Wiessner *et al.*, 1986), indicate involvement of membrane in calcium oxalate urolithiasis.

Erythrocytes, form a prototypal system for enquiries into the structure and functions of biological membranes and reflect the membrane alterations existing elsewhere in the body (Garay *et al.*, 1980). Two unique features of mammalian red cell, i.e., its discoid shape and ability to undergo passive deformation during passage through microvasculature, are maintained by the lipid bilayer and submembrane reticulum of membranous proteins (Goodman and Kathleen, 1983; Nakao *et al.*, 1990; Elgsaeter and Mikkelsen, 1991). Perturbations in the skeletal assembly are known to cause increased rigidity and irrecoverable membrane flow (Evans and Hochmuth, 1976), leading to cellular deformity and fragmentation (Palek and Jarolim, 1993).

Vitamin B_6 deficiency (Gershoff, 1970; Nath *et al.*, 1990) and excess consumption of refined sugars (Ribaya and Gershoff, 1984; Kaul *et al.*, 1993) have been shown to produce hyperoxaluria and nephrocalcinosis in experimental animals. Hyperabsorption of oxalate by the intestinal and renal brush border membrane in vitamin B_6 deficient rats (Gupta *et al.*, 1988), suggests an underlying defect in cellular transport of oxalate in vitamin deficient conditions. This was also demonstrated by significantly increased transmembrane oxalate flux observed in erythrocytes of vitamin B_6 deficient rats (Kaul *et al.*, 1993). Alterations in the erythrocyte oxalate transport have also been reported in idiopathic calcium oxalate stone formers (Baggio *et al.*, 1986; Narula *et al.*, 1991). This increased oxalate flux in the erythrocytes of stone

Table 1. Composition of the diet.

Ingredient	(g/100 g)	
Casein[1]	25.0	
Carbohydrates	23.0 (Sucrose) + 28.7 (Starch)	51.7: Galactose
Groundnut oil	15.0	
Salt mixture[2]	6.1	
Vitamin B_6-free Vitamin mixture[3]	2.2	

[1]Commercially available casein was made vitamin free by repeated washings with ethanol and water.
[2]Procured from SISCO Research Laboratory, Bombay.
[3]Prepared as described in ICN catalogue (Nutritional and Biochemical Division of ICN, Cleveland, Ohio)

formers has been speculated to be due to altered phosphorylation of band-3 protein (Baggio *et al.*, 1990, 1991). Increased band-3 protein phosphorylation is known to produce morphological changes in erythrocytes as reported in South-East Asian ovalocytosis (Jones *et al.*, 1991) and hereditary spherocytosis (Jarolim *et al.*, 1990).

Therefore, to investigate if functional alterations in erythrocytes, similar to ion transporting capacity, membrane bound enzymes (Selvam and Sumathi, 1987) or lipid peroxidation (Selvam and Ravichandran, 1991) observed in urolithic conditions, are also associated with changes in red cell shape and physical properties, the present study was conducted to determine alterations in morphology [using scanning electron microscopy (SEM)] and physical state (osmotic fragility) of erythrocytes in experimental hyperoxaluria, induced by feeding vitamin B_6 deficient diet with or without galactose to male rats. Studies were also performed to correlate these changes with erythrocyte membrane lipid composition.

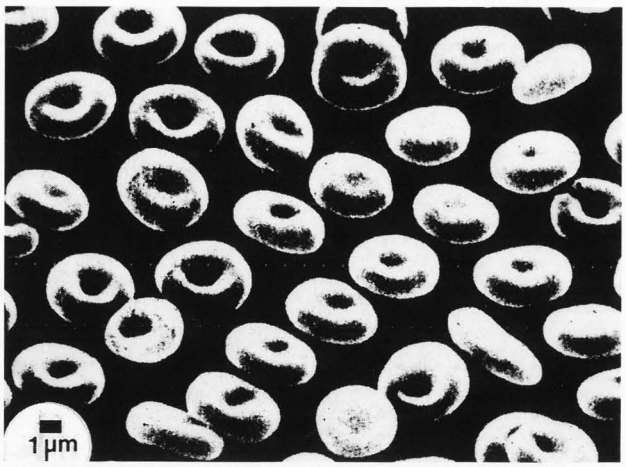
Materials and Methods

Male albino rats of Wistar strain (body weight 40-50 grams) were divided into four groups of 8 animals each.

Group A: Vitamin B_6 deficient: Fed *ad libitum* on vitamin B_6 deficient diet (Table 1).

Group B: Vitamin B_6 control: Pair-fed with group A with same diet supplemented with 24.0 mg pyridoxine HCl/kg diet.

Group C: Vitamin B_6 deficient + galactose: Fed *ad libitum* on vitamin B_6 deficient diet containing galactose (51.7%) as sole source of carbohydrate.



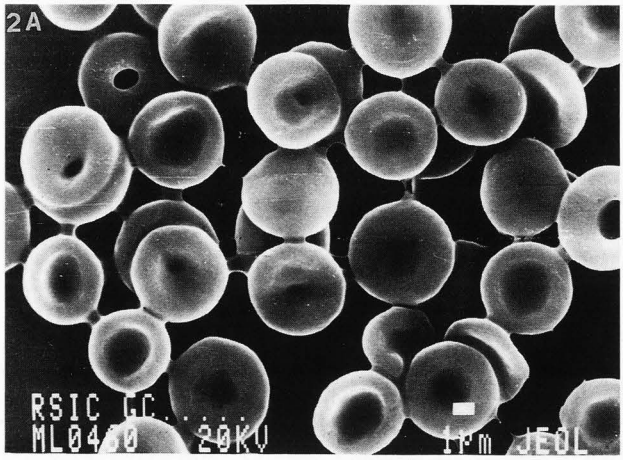
Figures 1 (above) and 2, 3 and 4 (on the facing page). Scanning electron micrographs of rat erythrocytes. **Figure 1.** Vitamin B_6 control group. **Figures 2A and 2B.** Vitamin B_6 deficient group. **Figures 3A and 3B.** Vitamin B_6 deficient + galactose group. **Figures 4A and 4B.** Galactose control group. Bars = 1 μm in all micrographs except Figure 3A, where bar = 10 μm.

Group D: Galactose control: Pair-fed with group C with the same diet supplemented with 24.0 mg pyridoxine HCl/kg diet.

Rats were fed their respective dietary regimen for a period of four weeks. At the end of experimental period, the clinical symptoms of vitamin B_6 deficiency, i.e., acrodynia and alopecia, were prominent in group A and C and the deficiency was biochemically confirmed by assaying erythrocyte alanine transaminase activity and its pyridoxal phosphate stimulation index (Kishi and Folkares, 1976). The animals were placed in metabolic cages for 24 hours urine collection, which was analysed for oxalate (Hodgkinson and Williams, 1972) and creatinine (Natelson, 1963). Blood was drawn into heparinised tubes from the optic sinus of rats under mild anaesthesia and processed for further studies.

Scanning electron microscopy

Scanning electron microscopy of red blood cells (RBC) was performed by the method of Dershwitz *et al.* (1985). Fresh RBC gently suspended in excess of 3% phosphate buffered glutaraldehyde (pH 7.4) were allowed to fix for 30 minutes. The preparation was washed twice with phosphate buffered saline (PBS) to remove excess glutaraldehyde and cells refixed with 1% osmium tetroxide. The cells were then dehydrated by step-wise exposure to increasing concentrations of ethanol (50% to absolute). The cells were exposed to each ethanol concentration for 5-10 minutes and centrifuged.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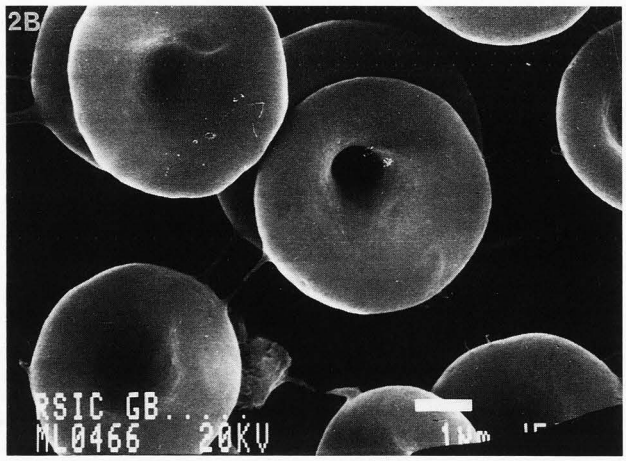
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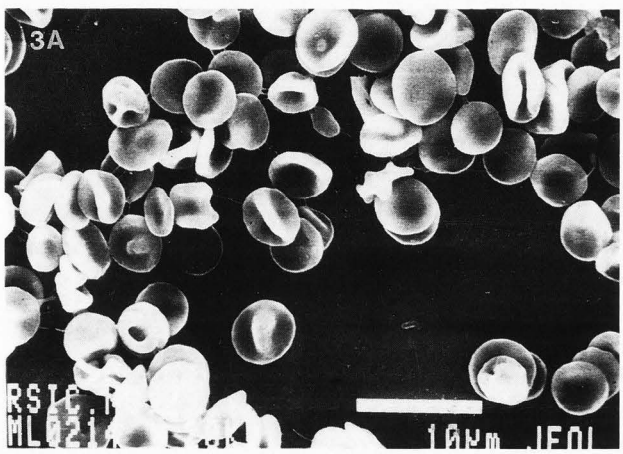
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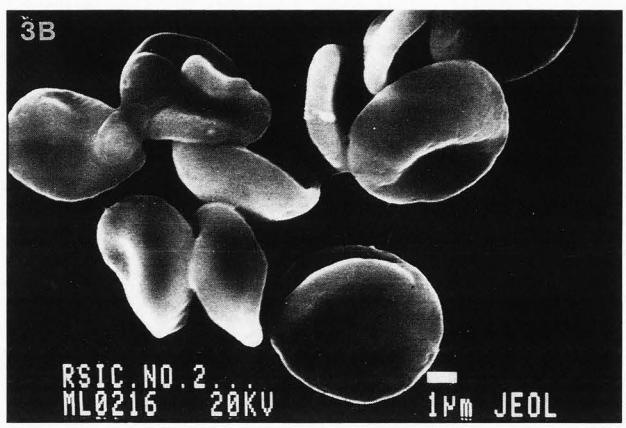
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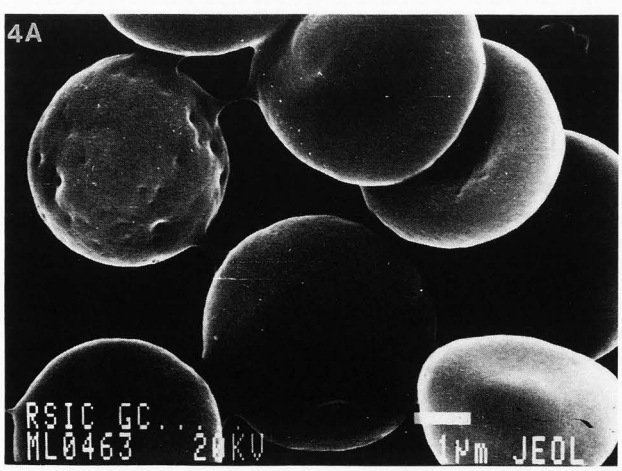
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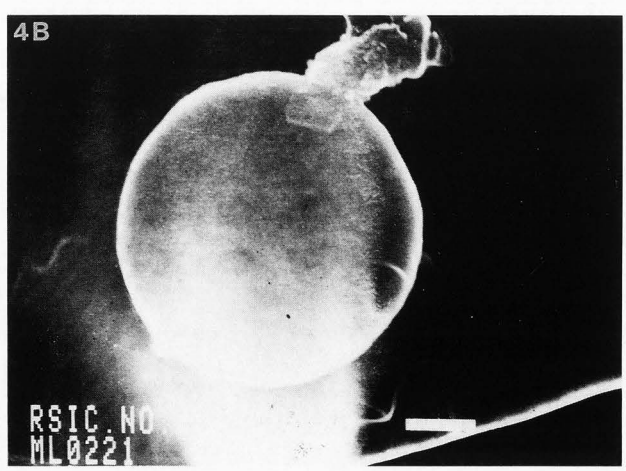
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Table 2. Effect of vitamin B_6 deficiency and/or galactose feeding on the osmotic-fragility of rat erythrocytes. Values are mean ± standard deviation (SD) of 8 observations.

Groups	MCF*	% Hemolysis at 4 g/l NaCl
A: Vitamin B_6 deficient	3.55 ± 0.32^{2}	19 ± 4.2^{3}
B: Vitamin B_6 control	4.08 ± 0.28^{ac}	55 ± 4.1^{ac}
C: Vitamin B_6 deficient + galactose	3.70 ± 0.36^{1}	33 ± 1.5^{3}
D: Galactose control	4.60 ± 0.29^{abc2}	73 ± 6.2^{abc3}

*MCF = Median corpuscular fragility: the concentration of saline causing 50% hemolysis.

$^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$ as compared to vitamin B_6 control (analysed by students unpaired t-test).

^{abc}Denote that the value is significantly higher from the value for the group represented by that letter, i.e., group A, B, C, respectively (analysis by ANOVA and Scheffe's test at $p < 0.05$).

Air-drying of the cells was avoided by keeping a small volume of ethanol above the pellet after each step. A few drops of cell suspension in absolute alcohol was put on a polished stub, air-dried, coated with gold (on a JFC 1100, sputter coater JEOL) and examined in JEM 1200 EX electron microscopy with ASID attachment (JEOL), operated at an accelerating voltage of 20 kV.

Osmotic fragility test

Osmotic fragility of erythrocytes was measured in terms of lysis in hypotonic saline by the method of Godal and Heist (1981). Fifty microliters of heparinized fresh blood was added to 10 ml PBS of varying concentrations (1-9 g/l NaCl). The tubes were kept at room temperature for 1.5 hours, remixed and centrifuged at 250 *g* for 15 minutes. Hemoglobin content of supernatant was read on a spectrophotometer at 540 nm. The amount of lysis in each tube was compared with that in 100% lysis tube (1 g/l NaCl).

Erythrocyte lipids

Lipids were extracted from the red cells by the isopropanol chloroform extraction procedure of Rose and Oklander (1965). Aliquots of the lipid extract were used

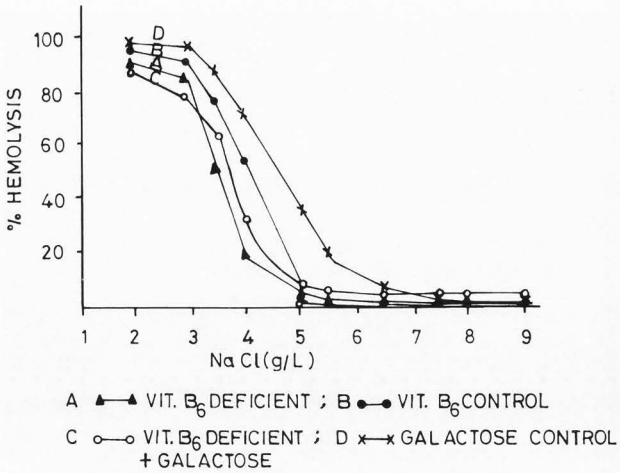

Figure 5. Effect of vitamin B_6 deficient, vitamin B_6 deficient + galactose, and their respective control diets on rat erythrocyte osmotic fragility. Values are mean ± SD of 8 observations. Osmotic fragility was determined using the continuous dilution method described in the text. The extent of hemolysis is plotted for different salt concentrations in g/l. The test tube containing the lowest buffer salt concentration (1 g/l NaCl) gives the level for 100% hemolysis. The curve for vitamin B_6 deficient (▲—▲) and vitamin B_6 deficient + galactose (o—o) groups are shifted to lower region, whereas galactose control group (x—x) curve is shifted to higher fragility region as compared to the vitamin B_6 control group curve (●—●).

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for colorimetric estimation of cholesterol (Zlatkis *et al.*, 1953), glycolipids (Dubois *et al.*, 1956) and total phospholipids (Marinetti, 1962). Different classes of phospholipids were separated by thin-layer chromatography (TLC) on silica gel-H using chloroform: methanol: ammonia (65: 25: 4 v/v) as the solvent system (Stahl, 1969). The separated phospholipids were visualized with iodine vapors, scraped from the TLC plates and analysed for lipid phosphorus.

Statistical analysis

Statistical comparison of two groups (treated and its respective control) was done by Student's unpaired t-test. For multiple comparisons, with a single control and for all possible comparisons, analysis of variance (ANOVA) and Scheffe's test was used. The difference was considered statistically significant when the probability of chance occurrence was < 0.05.

Results

After 4 weeks of feeding respective diets, vitamin B_6 deficiency was confirmed by a significant decrease in erythrocyte alanine transaminase activity in groups A

Table 3. Effect of vitamin B_6 deficiency and/or galactose feeding on erythrocyte membrane lipid composition (values are mean ± SD of 8 observations).

Groups	(mg/ml packed cells)				(molar ratio) Cholesterol/ Phospholipid
	Total lipids	Phospholipids	Cholesterol	Glycolipid	
A. Vitamin B_6 deficient	6.13 ± 0.57	3.88 ± 0.65	1.31 ± 0.09[2]	0.74 ± 0.12	0.68 ± 0.06[3]
B. Vitamin B_6 control	6.09 ± 0.62	3.56 ± 0.54	1.46 ± 0.11[a]	0.78 ± 0.09	0.83 ± 0.08[a]
C. Vitamin B_6 deficient + Galactose	6.38 ± 0.86	3.71 ± 0.63	1.42 ± 0.14	0.82 ± 0.08	0.78 ± 0.06
D. Galactose control	6.42 ± 0.41	3.40 ± 0.68	1.85 ± 0.11[abc3]	0.85 ± 0.09	1.12 ± 0.09[abc3]

[2]$p < 0.01$ and [3]$p < 0.001$ as compared to controls (analysed by Student's unpaired t-test).
[abc]Denote that the value is significantly higher from the value for the group represented by that letter, i.e., group A, B, C, respectively (analysed by ANOVA and Scheffe's test at $p < 0.05$).

and C ($P < 0.001$) and about 6-7 fold increase in pyridoxal phosphate stimulation index as compared to control groups (groups B and D). At the end of experimental period, significant hyperoxaluria was induced in vitamin B_6 deficient + galactose group (1.5 ± 0.022 mg oxalate/mg creatinine, $p < 0.001$), galactose control group (0.89 ± 0.15 mg/mg creatinine, $p < 0.001$) and vitamin B_6 deficient group (0.47 ± 0.12 mg/mg creatinine, $p < 0.01$) as compared to vitamin B_6 control group (0.31 ± 0.07 mg/mg creatinine).

Scanning electron microscopy

Figures 1 to 4 show the scanning electron micrographs of erythrocytes in various experimental groups. Figure 1 shows the normal biconcave cells (discocytes) in the vitamin B_6 control group (group B). In vitamin B_6 deficient rats, with or without galactose feeding (i.e., groups C and A, respectively), the majority of cells have abnormal morphology and the field depicts cup-shaped (stomatocytes), flattened (leptocytes) and other variously deformed cells (Figs. 2A, 2B, 3A and 3B). However, among the two vitamin B_6 deficient groups, extent of deformity is more pronounced in the vitamin B_6 deficient + galactose group (Figs. 3A and 3B), showing almost all the cells in deformed shapes (Figs. 3A and 3B), including a few pitted cells (Fig. 3A). Most of the cells in galactose control group are spherical in shape, i.e., spherocytes (Figs. 4A and 4B). The cells in this group have abnormal surface appearances, with some of the cells showing pitted surfaces.

Osmotic fragility

Figure 5 shows the erythrocyte osmotic fragility curves in vitamin B_6 deficient rats with and without galactose feeding and their respective controls. The osmotic fragility curves for vitamin B_6 deficient group and vitamin B_6 deficient + galactose fed group are shifted towards the left, whereas the curve for galactose control is shifted towards the right as compared to the osmotic fragility curve of normal rat erythrocytes (group B). Vitamin B_6 deficiency, with and without galactose feeding (groups A and C), significantly lowered the median corpuscular fragility (MCF: concentration of saline causing 50% hemolysis) in comparison to that of the vitamin B_6 control (Table 2). The galactose control group (group D) had a significantly higher MCF when compared to the control (group B). These results indicate a decreased erythrocyte osmotic fragility in pyridoxine deficiency (groups A and C) and increased osmotic fragility by galactose feeding (group D). This is also evident from the lower percentage of hemolysis at 4 g/l NaCl concentration in vitamin B_6 deficient groups (groups A and C) and the higher percentage hemolysis in the galactose control group (group D) than in the vitamin B_6 control group (group B) (Table 2).

Erythrocyte membrane lipids

Erythrocyte membrane lipid analysis depicted no significant change in total lipid, total phospholipid and glycolipid content in any of the groups studied (Table 3). However, the cholesterol content was significantly increased ($p < 0.001$) in the galactose control group (group D) and decreased ($p < 0.01$) in the vitamin B_6 deficient group (group A) when compared with the vitamin B_6 control group (group B). Erythrocyte membrane cholesterol content was observed to be maximum in the galactose control group (group D). The molar cholesterol-to-phospholipid ratio was significantly increased by 35% in the galactose control group (group D) and decreased by about 18% in the vitamin B_6 deficient group (group A) and 6% in the vitamin B_6 deficient + galactose fed group (group C) in comparison to the vitamin B_6 control group (group B) (Table 3).

Table 4. Effect of vitamin B_6 deficiency and/or galactose feeding on various phospholipid fractions in rat erythrocyte membranes (values are mean ± SD of 8 observations).

GROUPS	% of total phospholipids				
	Lysophosphatidyl choline (LPC)	Sphingomyelin (SM)	Phosphatidyl-choline (PC)	Phosphatidyl-serine (PS) + Phosphatidyl-inositol (PI)	Phosphatidyl-ethanolamine (PE)
A. Vitamin B_6	3.1 ± 1.4	17.8 ± 2.4[1]	35.6 ± 4.1	18.0 ± 3.8	25.3 ± 2.3[b2]
B. Vitamin B_6 control	3.0 ± 1.5	21.0 ± 2.8	34.4 ± 3.6	19.6 ± 3.6	22.0 ± 2.0
C. Vitamin B_6 deficient + galactose	2.8 ± 1.2	20.0 ± 2.2	34.0 ± 3.1	18.5 ± 3.2	24.7 ± 2.1[b1]
D. Galactose control	2.0 ± 1.0	25.0 ± 3.1[abc1]	31.6 ± 3.5	18.4 ± 3.0	23.3 ± 3.0

[1] $p < 0.05$ and [2] $p < 0.01$ as compared to vitamin B_6 control group (as analysed by Student's unpaired t-test).
[abc] Denote that the value is significantly higher from the value for the group represented by that letter, i.e., group A, B, C, respectively (analysed by ANOVA and Scheffe's test at $p < 0.05$).

Among individual phospholipids (Table 4), sphingomyelin (SM) was significantly less ($p < 0.05$) in the vitamin B_6 deficient group (group A) and significantly higher ($p < 0.05$) in the galactose control group (group D) than in the vitamin B_6 control group (group B). The SM content of erythrocyte membrane was higher in the galactose control group (group D) than in any other of the three groups ($p < 0.05$, analysed by Scheffe's test). When compared to that of the vitamin B_6 control (group B), phosphatidylethanolamine (PE) content was significantly higher in both the vitamin B_6 deficient group ($p < 0.001$) and the vitamin B_6 deficient + galactose fed group ($p < 0.05$). Levels of other phospholipid fractions, viz., phosphatidylcholine (PC), phosphatidylserine + phosphatidylinositol (PS + PI) and lysophosphatidylcholine (LPC), did not show any significant change in any of the experimental groups.

Discussion

The normal erythrocyte is capable of maintaining the discoid shape unstressed throughout its life-span. However, under certain instances, in which biochemical alterations lead to weak skeleton, applied stresses in circulation result in membrane fragmentation and generation of spherocytes and other poikilocytes (Palek and Jarolim, 1993). Although the maintenance of shape has been mainly ascribed to spectrin-actin network underlying the erythrocyte membrane (Beaven *et al.*, 1990; Nakao *et al.*, 1990), lipids and other integral proteins of the cell membrane are also known to contribute to shape maintenance (Elgsaeter and Mikkelsen, 1991; Palek and Jarolim, 1993). Alterations in erythrocyte anion transporter (an integral membrane protein) have been reported in calcium oxalate stone formers (Borsatti, 1991) and hyperoxaluric animals (Kaul *et al.*, 1993). The present study has revealed significant changes in membrane lipid composition in erythrocytes of animals fed pyridoxine deficient diet with and without galactose to produce hyperoxaluria. These alterations are probably contributing towards morphological changes observed by SEM. Cholesterol depletion has been shown to induce discocyte to stomatocyte transformations (Chailley *et al.*, 1981; Lange *et al.*, 1982) which is in accordance with the results of the present study showing stomatocyte formation in vitamin B_6 deficient group (having reduced red cell membrane cholesterol levels) and spherocyte formation in the galactose control rats (which have significantly higher membrane cholesterol content). In proportion to their cholesterol-to-phospholipid ratio, discocyte-to-stomatocyte transformation was more pronounced in vitamin B_6 deficient group than in vitamin B_6 deficient + galactose group. Lange *et al.* (1980) proposed that cholesterol constraints the membrane contour against invagination leading to morphological alterations. The effect of membrane cholesterol levels on erythrocyte shape have also been suggested to be mediated through altered protein and lipid phosphorylation by membrane bound kinases (Chailley *et al.*, 1981). The erythrocyte shape abnormalities are also produced because of the asymmetry in distribution of membrane lipids between the two halves of RBC lipid bilayer (Daleke and Huestis, 1989). Higher sphingomyelin content (primarily found in the outer leaflet; Devaux, 1992) in galactose control group (group D), and higher phosphatidylethanolamine (present in the inner leaflet of the bilayer) in vitamin B_6

deficient groups (groups A and C), is also probably contributing to spherocytic and stomatocytic transformations observed respectively in these groups. Selective insertion or removal of phospholipids from inner or outer membrane leaflets has been shown to induce echinocytic or stomatocytic transformations (Lange and Slayton, 1982). In addition to alterations in shapes, surface pits were observed in vitamin B_6 deficient + galactose (group C) and galactose control (group D) groups. Such surface pits have been reported in neonatal red cells and splenectomized adult red cells (Matovcik and Mentzer, 1985) and are described as sites of formation of endocytic vacuoles (Holroyde *et al.*, 1969). Scanning electron micrographs of vitamin B_6 deficient + galactose fed rats (Figs. 3A and 3B) showed the majority of cells in deformed shapes, which may also be because of reduced cellular deformability resulting from increased membrane rigidity probably due to a very high lipid peroxidation observed in erythrocytes of this group (unpublished observation). Membrane rigidity is a primary cause of reduced cell deformability (Yip *et al.*, 1983) and erythrocytes treated with hydrogen peroxide have been shown to have reduced cell deformability due to peroxide induced membrane rigidity (Synder *et al.*, 1988).

Osmotic fragility used as a measure of the physical state of the cell-membrane is related to changes in both the structural proteins and composition of the lipid bilayer. The shape of the erythrocyte also reflects its osmotic properties. The biconcave disc of the normal erythrocyte creates an advantageous surface area to volume relationship, allowing the cells to undergo marked deformation while maintaining the constant surface area. Greater osmotic fragility of erythrocytes in the galactose control group (group D) than of normal erythrocytes can be explained both on the basis of increased membrane cholesterol and spherocytic shape. Cholesterol rich erythrocytes have been reported to have increased osmotic fragility (Uysal, 1986). Also, the spherocytic cells have an increased volume to surface area, and thus, have a limited ability to take up water before causing osmotic lysis. Similar increases in red cell osmotic fragility have been noticed in patients with hereditary spherocytosis (Becker and Lux, 1992). Erythrocytes of vitamin B_6 deficient and vitamin B_6 deficient + galactose groups were osmotically more resistant than normal cells, which is due to their stomatocytic (cup-shaped) and leptocytic (flattened) appearance, which gives them a lower volume compared to surface area. Flattened or oval erythrocytes of patients of South-East Asian ovalocytosis (Saul *et al.*, 1984) and magnesium deficient rats (Tongyai *et al.*, 1989) have also been shown to have decreased osmotic fragility.

Thus, the results of the present study indicate that the altered red cell morphology and osmotic fragility in hyperoxaluric, vitamin B_6 deficient and/or galactose fed rats is predominantly due to the altered membrane lipid composition.

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Discussion with Reviewers

P.O. Schwille: What is the impact of higher than normal oxalate blood level on damage to transporting epithelia, isolated cells such as in blood?

Authors: Although there are no reports available which directly correlate the blood oxalate levels to membrane damage, moderate increase in urinary oxalate has been shown to damage renal tubular epithelium as assessed by urinary excretion of tubular enzyme N-acetyl-ß glucuronidase (Khan *et al.*, 1989). Increased levels of lipid peroxidation in erythrocyte membranes have been reported in kidney stone formers (Anuradha and Selvam, 1989) suggestive of membrane alterations by increased oxalate levels.

P.O. Schwille: Is there evidence from the literature that oxalate itself or oxalate-dependent processes interfere with carriers such as the Na^+/H^+ antiporter, Cl^-/HCO_3^- exchanger, etc.?

Authors: Studies in our laboratory (Kaul *et al.*, 1994) on erythrocytes treated with isothiocyanoderivative DIDS (a potent inhibitor of band-3 transport system), exhibited a significant inhibition (about 40-60%) of oxalate uptake, implying that major bulk of oxalate transport in erythrocytes is mediated via band-3-anion transporter which is known to mainly mediate Cl^-/HCO_3^- exchange. Inhibition of erythrocyte oxalate flux by DIDS has also been reported in many idiopathic stone formers (Baggio *et al.*, 1984).

In non-erythroid cells, several distinct transporters functionally related to band-3 have been reported (Aronson, 1989). In rat colon mucosa, oxalate and chloride share a common transport pathway and the Cl^- exchanges with HCO_3^- (Hatch *et al.*, 1984). In rat renal brush border membrane (BBM), oxalate has been shown to be transported via Na^+-SO_4^{2-} co-transport system (Bastlein and Burckhardt, 1986), as well as by oxalate: OH^- exchange via the anion transport system (Yamakawa and Kawamura, 1990). In the rabbit proximal tubular BBM, a chloride (formate)/oxalate exchanger has been identified (Karninski and Aronson, 1987).

Reviewer IV: When comparing the oxalate:creatinine ratio with visual morphological changes, small changes in membrane composition and structure seem to occur even at intermediate levels of oxalate. The cells in Figures 4A and 4B (group D) have the most abnormal shapes. The cells from group D shown in Figure 4A is pitted, while there is debris in Figure 4B. Oxalate levels in group D are two to three times higher than those found in control groups A and B. Group C is almost twice as hyperoxaluric as even group D.

Authors: Pyridoxine deficiency in rats produces significant hyperoxaluria and is also known to cause a generalized alteration at the membrane level indicated by increased oxalate uptake by intestinal and renal brush border membrane (Gupta *et al.*, 1988) and enhanced oxalate flux in the erythrocytes (Kaul *et al.*, 1993). Hyperoxaluria in galactose fed rats (group D) is mainly due to increased endogenous synthesis of oxalate, whereas highest urinary oxalate levels observed in pyridoxine deficient rats fed galactose (group C), were due to cumulative effect of both enhanced endogenous synthesis and membrane alterations (Kaul *et al.*, 1993). As observed in the present paper, vitamin B_6 deficiency and galactose feeding lead to distinct alterations in membrane composition (Tables 3 and 4) hence producing different alterations in shape and osmotic fragility even though both cause hyperoxaluria.

In our previous paper (Kaul *et al.*, 1993, to which the reviewer has referred), it was observed that increase in urinary oxalate does not concurrently increase the transmembrane oxalate flux in erythrocytes; this was also reported by other investigators (Baggio *et al.*, 1986; Motola *et al.*, 1992). It was suggested that increased erythrocyte flux is observed only when the defect is at the membrane level and not in all cases of idiopathic calcium oxalate lithiasis.

Please see next page for additional references.

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