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ERYTHROCYTE MEMBRANE ATPASES: EFFECT OF HYPERPHOSPHATAEMIA ON ATPASES

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ABSTRACT

The levels of Na^+, K^+ and Ca^{2+}-ATPases found in the erythrocyte membrane of sickle cell patients were significantly lower than normal. Sickle cell erythrocyte membrane Mg^{2+}-ATPase activity was found to be higher than normal. In sickle cell anaemia patients (HbSS), hyperphosphataemia was observed in infancy (0 - 5 yrs) and early childhood (6 - 10 yrs). Na^+, K^+-ATPase activity increased in both normal and sickle cell patients except for the age group 6 - 10yrs.

Exogenous inorganic phosphate inhibited Na^+, K^+ATPase in both normal and sickle cell erythrocyte membrane by 24.0% and 33.0% respectively. The difference in activity of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the presence and absence of phosphate was not statistically significant.
A relationship was found between the plasma phosphate level and Na^+, K^+-ATPase activity and this may provide an explanation for the low Na^+, K^+-ATPase activity found in sickle cell patients.

Key words: Hyperphosphataemia, ATPase, Erythrocyte membrane, inorganic phosphate, sickle cells.

INTRODUCTION

The stability of the erythrocyte membrane is achieved by the unidirectional activated transport of ions (Kyte, 1971). The red cell membrane consists of three different adenosine triphosphatases (Drickamer, 1974). These enzymes (Na^+, K^+, Ca^{2+}-ATPase) are responsible for the vectorial transport of ions. Since active transport is energy dependent, there was need to carry out a study on erythrocyte membrane ATPase of normal and sickle cells.

Inorganic phosphate (Pi) has long been known to play an important role in biological systems. Na^+, K^{2+}-ATPase activity has been shown to be inhibited by Pi, a product of ATP hydrolysis (Hesketh et al, 1978). Although there has been great interest in phosphate homeostasis in recent years, little work has been done on corresponding intracellular levels and their changes in disease states. There are, however, many diseases in man in which the plasma phosphate concentration is altered (Knockel, 1977). In clinical disorders where phosphate metabolism is changed, only the extracellular pi (Plasma Pi) has been studied in detail.

The study carried out by knockel (1977) shows that a reduction in the plasma phosphate is accompanied by a similar change in the intracellular Pi concentration. In an attempt to explain the low Na^+, K^+-ATPase activity observed in sickle erythrocyte membrane, a study on the effect of exogenous Pi on ATPase activity was carried out. Plasma phosphate levels were monitored in both normal and sickle blood and ATPase activities were assayed in the presence and absence of exogenous inorganic phosphate.

MATERIALS AND METHODS

Blood Collection

venous blood samples were obtained from 335 patients of both sexes, attending the University of Port Harcourt Teaching Hospital and Medical Centre, University of Port Harcourt. The homozygous sickle condition of these patients was confirmed by electrophoresis.

The blood samples were collected into heparinized tubes. Blood samples from normal individuals were collected in a similar manner. The normal subjects were aged 0 - 55 years. It was not logistically possible to obtain HbSS blood from patients beyond 30 years of age.

Preparation

The erythrocyte ghost cells were prepared based on the method of Hamlyn and Duffy (1978). Red cells from fresh human heparinized venous blood were washed three times with 0.15M NaCl pH 7.4. Membrane was prepared by hypotonic haemolysis in

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swirling ice-cold 5mM NaH_2PO_4 pH 7.7. The mixture was centrifuged at 5,000g for 1hr. The supernatant was removed and ghosts were later suspended in 3.0 ml distilled water. The membranes were stored at 4°C for not more than 12 hr. before being assayed for enzyme activity.

Assay of ATPase Activity

Membrane ATPase Activities were estimated by the procedure described by Hesketh et al., (1978). ATPase activity was assayed by measuring the inorganic phosphate released from ATP hydrolysis. Inorganic phosphate released in the ATPase assay was determined by the method of Fiske and Subbarow (1925). The activity of the enzyme was expressed as µmole Pi/mg protein/h. Protein was estimated by the method of Lowry et al, (1951).

Na^+, K^+-ATPase

The enzyme catalyzed reaction was followed spectrophotomertically at 725nm in a mixture containing 0.5ml 350mM NaCl, 0.5 ml, 17.5mM KCL, 0.5 ml 21.0mM $MgCl_2$, 0.5ml, 10mM Tris-HCl buffer pH 7.4 and 0.5 ml 2mM ATP-Na_2. Reaction was started by adding 0.20ml ghost cells. The mixture was incubated for 1 hr. at 37oC. Reaction was terminated by adding 0.8 ml ice-cold 10% TCA. Tubes were then allowed to stand at 4°C for 20 minutes after which they were centrifuged at 4,000g for 5 min. To the supernatant was added 1.0ml of 2.5% ammonium molybdate and after 10 min. 1.0 ml 2.0% ascorbic acid. The tubes were then allowed to stand for 20 min for colour to develop. Absorbance was read at 725nm using Spectronic 21 spectrophotometer.

Ca^{2+}-ATPase

The assay medium contained 0.5 ml 21 mM $MgCl_2$, 0.5 ml 17.5mM $CaCl_2$, 0.5 ml 10mM Tris-HCL buffer pH 7.4 and 0.5 ml 2.0mM ATP-Na_2. The reaction was started and terminated as already described for Na^+, K^+-ATPase.

Mg^{2+}-ATPase

Mg^{2+}-ATPase activity was assayed spectrophotometrically in a medium containing 0.5 ml 21mM $MgCl_2$, 0.5mM ATP-Na_2, 0.5 ml 10mM Tris-HCl buffer pH 7.4 and 1.0 ml distilled water and usually 0.2 ml ghost cells. The inorganic phosphate liberated was measured by the method of Fiske and Subbarow (1925).

Determination of Protein

The protein content of erythrocyte membrane preparations was determined by the method of Lowry et al, (1951), with bovine serum albumin as a standard.

Plasma Phosphate Determination

The method used for measuring inorganic phosphate in plasma was based on the phosphate method of Hall (1963). Blood samples were obtained from HbAA, HbAS and HbSS subjects and put into sterile heparinized tubes. The contents were thoroughly mixed. Plasma were deproteinized by precipitation with 0.8 ml ice-cold 10% trichloroacetic acid and centrifuged at 4,000g for 15 min. 0.2 ml deproteinzed plasma was used in the experiments. The reaction mixture contained 0.5 ml 10mM Tris-HCl buffer pH 7.4, 0.2 plasma; and 1.0 ml 2.5% ammonium molybdate. After 10 min. 1.0 ml 2% ascorbic acid was then added. The tubes were allowed to stand for 20 min. for colour to develop. Optical density (OD) was read at 725nm using a spectronic 21 spectrophotometer. A standard curve was set up using 1.0M NaH_2PO_4 standard solution (0 - 100 µmoles).

Effect of Exogenous Inorganic Phosphate on ATPase Activity

ATPase (Na^+, K^+,Ca^{2+}- and Mg^{2+}) activity was assayed as already described in the presence of 1 - 5mM NaH_2PO_4. It was found that at 2mM NaH_2PO_4 there was inhibition of the enzyme in all the three genotypes. Therefore, this concentration was used in the experiments using NaH_2PO_4 as inhibitor.

Table 1: Na^+, K^+-ATPase activity* in HbAA/HbAS subjects of different ages

Age (yr)	ATPase (µmole Pi/mgprotein/h) Activity x 10^{-3}	
Age (yr)	HbAA	HbAS
0 - 10 (n = 10)	242.0 ± 1.9	228.2 ± 2.0
11 - 20 (n = 10)	268.5 ± 4.5	274.2 ± 3.2
21 - 30 (n = 10)	291.2 ± 3.1	288.8 ± 2.2
31 - 40 (n = 10)	346.0 ± 7.5	328.4 ± 4.0
41 - 50 (n = 10)	396.0 ± 6.5	389.3 ± 4.2
>50 (n = 10)	403.3 ± 2.9	401.4 ± 1.8

*values are means ± standard deviations (n=10). The number of samples analyzed is indicated in parentheses.

RESULTS

ATPase (Na^+,K^+, Ca^{2+}- and Mg^{2+}) activities of sickle and normal RBC membrane are shown in fig. 1. Ghosts from AA cells exhibited a Na^+, K^+-ATPase activity of 284.7 ± 20.2 x 10^{-3} µmole Pi/mg protein/h). Ghosts from SS cells had a lower activity (118.0 ± 2.8 x 10-3 µmole Pi/mg/h) than AA ghosts. The Na^+, K^+-ATPase activity of AS cells was not significantly different from that of AA RBCs. ($P \leq 0.05$). The Ca^{2+}-ATPase activity of sickle cell membrane is lower than normal. The activity of the enzyme in sickle erythrocytes is about 71.0% of the normal. Mg^{2+}-ATPase activity of sickle cell membrane is higher than normal. Ghosts of AA cells exhibited a membrane activity of 144.8 ± 1.3 x 10^{-3} µmole Pi/mg/h as compared to SS cells with 234.4 ± 2.0 x 10^{-3} µmole Pi/mg/h.

Na^+, K^+-ATPase activity increased with age up to 50 yr. in AA and AS cells and up to 30 yr. in SS cells (Fig. 2). In AA and AS cells after 50 yr., there was no significant ($P < 0.05$) increase in Na^+, K^+-ATPase activity (Table 1). It was not possible to get HbSS cells beyond 30 yr. The activities of Ca^{2+}-Mg^{2+} ATPase increased only marginally with age (Figs 3 and 4). The difference in activities between the various age groups is not statistically significant ($P < 0.05$).

Plasma Inorganic Phosphate Determination

Plasma phosphate level in normal and sickle cell patients is shown in Table 2. In sickle cell anaemia patients, HbSS hyperphosphataemia was observed in infancy (0 - 5yr) and early childhood (6 - 10 yr). When compared to normal subjects, patients with sickle cell anaemia exhibited raised plasma phosphate concentrations. Plasma Pi values measured in heterozygous (HbAS) individual were very close to the ones obtained for normal subjects (HbAA). Fig. 5 shows the effect of age on plasma phosphate levels in

Table 2: Plasma phosphate levels in normal and sickle cell patients*

Plasma	Age (yr)	Inorganic phosphate (µmole/l)
HbAA (25)		1205.0 ± 20.5
HbAS (25)	0-5	1195.5 ± 26.0
HbSS (20)		2514.0 ± 47.5
HbAA (25)		1374.0 ± 20.0
HbAS (25)	6 - 10	1264.0 ± 19.0
HbSS (20)		2814.5 ± 40.0
HbAA (25)		893.0 ± 8.5
HbAS (25)	11 - 15	901.0 ± 12.5
HbSS (20)		1706.0 ± 65.0
HbAA (25)		761.5 ± 6.0
HbAS (25)	16 - 20	741.0 ± 15.0
HbSS (20)		1128.5 ± 28.0
HbAA (25)		627.5 ± 7.5
HbAS (25)	21 - 25	624.0 ± 11.0
HbSS (20)		723.5 ± 17.0

*Values are means ± standard deviations of triplicate determinations. The number of samples analyszed is given in parentheses.

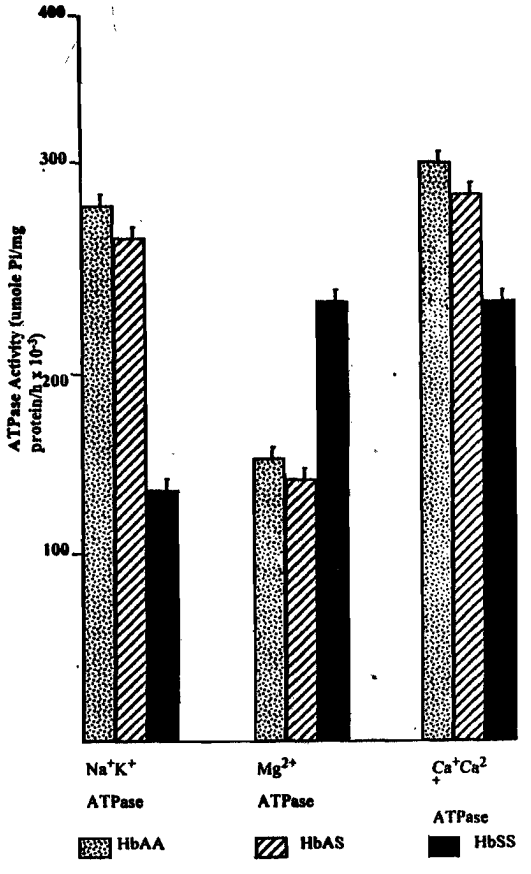

Fig. 1 Erythrocyte Membrane ATPase

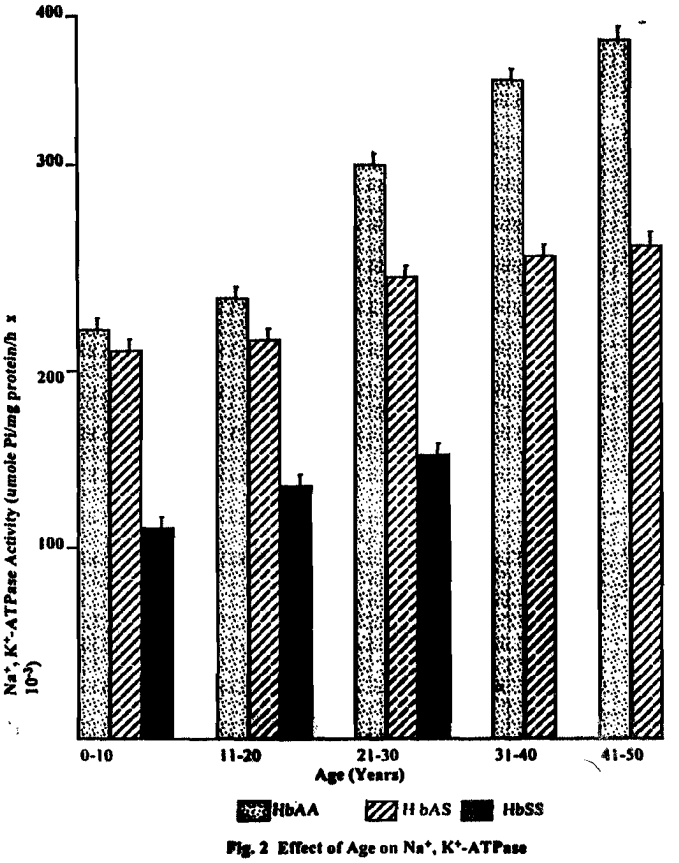

Fig. 2 Effect of Age on Na^+, K^+-ATPase

Table 3: Effect of Exogenous inorganic phosphate (NaH_2PO4) on erythrocyte membrane ATPases*

Membrane	ATPases						
	Mg^{2+} - Without NaH_2PO_4	Mg^{2+} - with NaH_2PO_4	Ca^{2+} - without NaH_2PO_4	Ca^{2+} - with $N_aH_2PO_4$	Na^+, K^+ - without NaH_2PO_4	Na^+, K^+ - with NaH_2PO_4	% inhibition (Na^+, K^+-ATPase)
sickle (n = 10)	220.2	216.1	218.2	214.2	180.0	120.5	33.0
	± 1.2	± 0.8	± 4.0	± 4.0	± 4.0	± 5.0	
Normal (n = 10)	128.4	127.2	316.2	312.2	282.2	214.6	24.0
	± 2.0	± 0.6	± 4.0	± 4.0	± 2.2	± 1.2	

*Values are means ± standard deviations. (n = Number of blood samples analyzed).

0.50 ml 2.0mM NaH_2PO_4 was used as inhibitor.

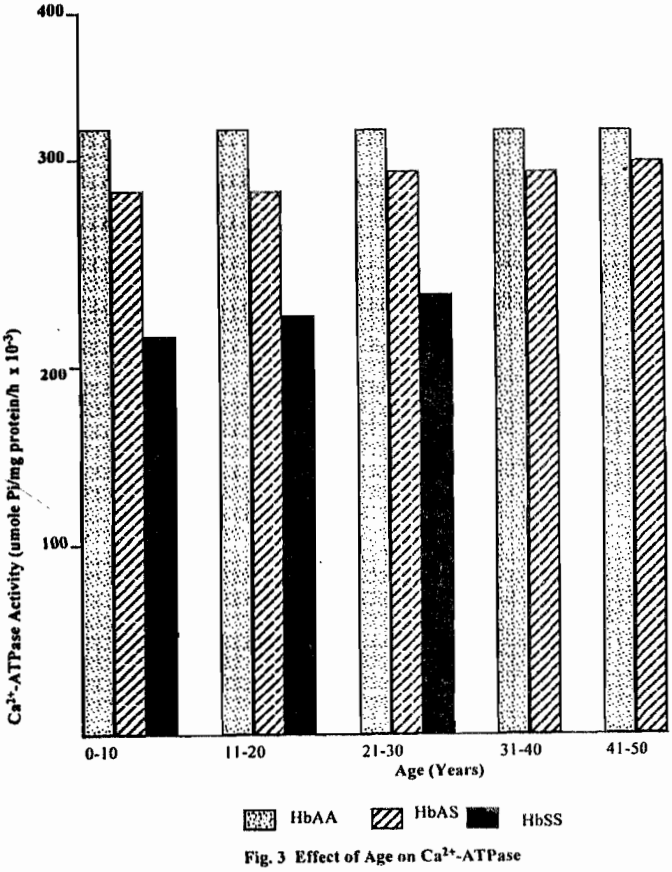

Fig. 3 Effect of Age on Ca^{2+}-ATPase

normal and sickle cell subjects. Plasma phosphate level decreased as age increased after the age of 10 years. After 20 years of age, HbSS Pi values declined rapidly. From 20 yr. and above no significant difference was observable between normal and sickle cell individuals.

Effect of Exogenous Pi on ATPase Activity

The effect of different concentrations (1 - 5mM) of NaH_2PO4 on Na^+-ATPase activity in normal and sickle cells is shown in Fig. 6. 2mM NaH_2PO_4 was found to give the maximum inhibition in all the three genotypes. Table 3 shows inhibition of Ca^{2+} and mg^{2+}-ATPase by 2mM NaH_2PO_4 in AA, AS and SS ghosts respectively.

The difference in activity of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the presence and absence of 2mM NaH_2PO_4 was not statistically significant Na^+, K^+-ATPase activities in SS and AA erythrocyte membranes were inhibited by 33% and 24% respectively.

DISCUSSION

Recent studies have shown that there are many changes in the sickle cell erythrocyte membrane (Riggs and Ingram, 1977). There are also several reports showing that the Ca^{2+} content of sickle cells is higher than that of normal red blood cells (Palek, 1973, Wiley

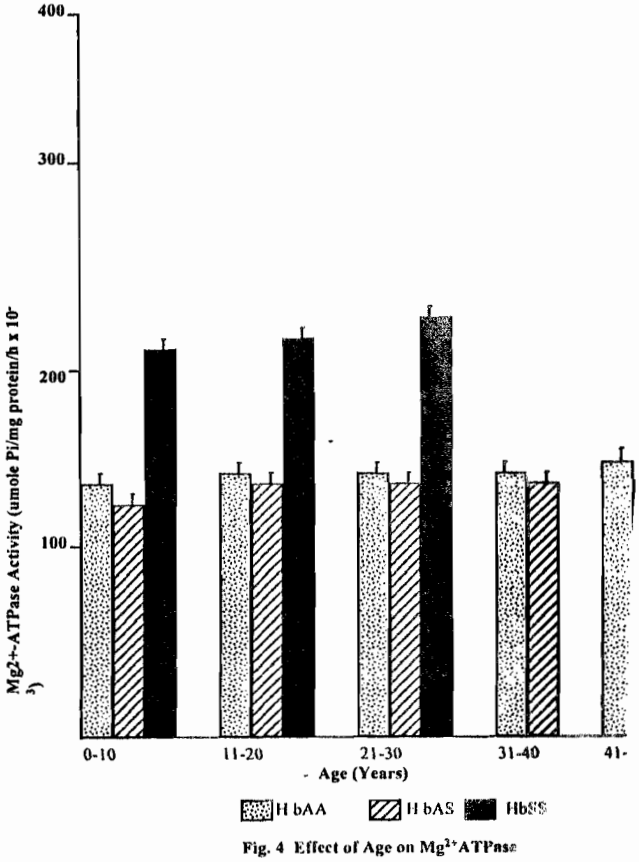

Fig. 4 Effect of Age on Mg^{2+}ATPase

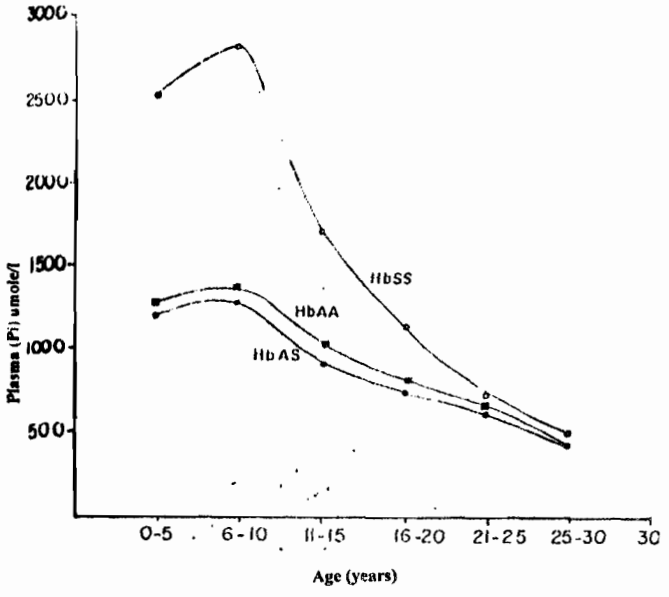

Fig. 5 Effect of Age on Plasma Phosphate Level in Normal and Sickle Cell Patients.

and Shaller, 1974, Palek, et al 1976). Bookchin and Lew (1980) showed that Ca^{2+}-ATPase activity of sickle cell erythrocyte membrane, a biochemical expression of the calcium pump is lower than normal. The findings of this study agrees with that of Bookchin and Lew (1980). The Ca^{2+}-ATPase activity of sickle cell erythrocyte membrane (Fig. 1) obtained in this study agrees with the data of Gopinath and Vincenzi (1979).

It has been observed in this study that there is a significant difference (P> 0.05) in the specific activity

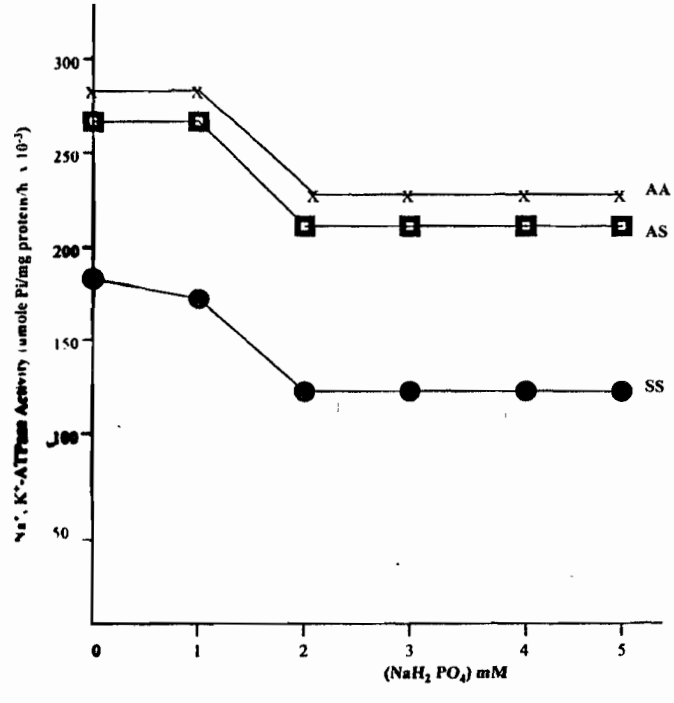

Fig. 6: Effect of inorganic Phosphate (NaH_2PO_4) mM on Na+, K+-ATPase activity in normal and Sickle cells.

of Na^+, K^+-ATPase for normal and sickle cells. This is at variance with the observation made by Gopinath and Vincenzi (1979). The activity of Na^+, K^+-ATPase of sickle erythrocyte membranes was found to be less than 50% of the normal. The low Na^+, K^+-ATPase activity of sickle erythrocyte membranes could be attributed to the high plasma inorganic phosphate observed in sickle cell patients. Na^+, K^+-ATPase activity has been shown to be inhibited by inorganic phosphate observed in sickle cell patients. Na^+, K^+-ATPase activity has been shown to be inhibited by inorganic phosphate, a product of ATP hydrolysis (Hesketh et al, 1978). We have demonstrated a state of hyperphoshataemia at the age of 0 - 20 yr in sickle cell disease.

There is no doubt that hyperphosphataemia is prevalent in sickle cell disease between the age 0 - 20yr, inorganic phosphate concentration being about two fold that for HbAA and HbAS subjects. Such high plasma phosphate level in sickle cell disease must surely have its implication in the overall phosphate metabolism in the sickler, particularly on bone mineralization as well as calcium conservation. It is significant that inorganic phosphate value returns to normal beyond 20yr of age. This is also the period when the rate of human bone formation begins to slow down. An increase in alkaline phosphatase activity is also well recognized in normal childhood, reflecting an expected increase in osteoblastic activity (Zilva and Pannall, 1979).

The finding of significantly higher Mg^{2+}-ATPase activity in sickle cell membranes compared to normals agrees with that of Niggli et al (1982) and Bewaji et al (1985). Mg^{2+}-ATPase is associated with permeation of phosphates and sugars across the membrane (White et al, 1978). The observation of high plasma inorganic phosphate in sickle cell patients reported here (Table 2) may be due to high activity of Mg^{2+}-ATPase also observed in them. Ca^{2+} affects Mg^{2+} ATPase and erythrocyte shape. At low Ca^{2+} concentration, activity is low but with higher concentration activity rises up to a maximum (Bewaji el at 1985).

It therefore seems possible that the high Mg^{2+}-ATPase activity reported for HbSS erythrocyte membranes could be due to the accumulation of Ca^{2+} by these cells (Bookchin and Lew, 1980).

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