GLOBAL JOURNAL OF MEDICAL SCIENCES VOL. 2, NO. 2: 2003: 107 - 114
COPYRIGHT(C) BACHUDO SCIENCE CO. LTD. ISSN 1596 - 2911. PRINTED IN NIGERIA.

STUDIES ON THE EFFECT OF AQUEOUS EXTRACTS OF *GARCINIA KOLA* SEEDS ON HUMAN ERYTHROCYTES ADENOSINE TRIPHOSPHATATSES OF HbAA, HbAS AND HbSS GENOTYPES

I. ELEKWA, M. O. MONANU and E. O. ANOSIKE

(Received 18 November 2002; Revision accepted 10 March 2003)

ABSTRACT

The activities of the membrane-bound ATPases (Na^+, K^+-ATPase: E. C. 3.6.1.37; Ca^{2+}-ATPase: E. C. 3.6.1.38, and Mg^{2+}-ATPase: E. C. 3.6.1.3) were determined in erythrocytes obtained from HbAA, HbAS and HbSS genotypes. For Na^+, K^+-ATPase and Ca^{2+}-ATPase, the activity trend was HbAA > HbAS > HbSS while for Mg^{2+}-ATPase, the trend was HbSS > HbAS > HbAA. The aqueous extract of Garcinia kola seed increased the activity of Na^+, K^+-ATPase and Ca^{2+}-ATPase in the three genotypes with the trend of HbSS > HbAS > HbAA. Phenylalanine, a known anti-sickling agent, showed similar activating effect as the extract. In contrast, both the extract and phenylalanine decreased the activity of Mg^{2+}-ATPase from all the genotypes, showing the trend of HbSS > HbAS > HbAA. The three ATPases obeyed Michaelis-Menten kinetics. The apparent K_m for the three different genotypes for ATP were similar for the ATPases with the values of 5.62±0.02, 5.27±0.02 and 4.65±0.03 mM ATP-Na_2 for (Na^+, K^+)- ; Ca^{2+}-; and Mg^{2+}-ATPases, respectively. An activation constant of 0.317 g/ml of extract, for the Ca^{2+}-ATPase of HbSS erythrocyte was obtained while the inhibition constant, K_i, of the extract on Mg^{2+}-ATPase from HbSS erythrocyte by the extract was 1.23 g/ml of extract. These findings are of significance to the suggested use of the aqueous extract of G. kola seeds in ameliorating sickle cell crisis.

Key words: adenosine triphosphatases, erythrocytes, genotypes, phenylalanine

INTRODUCTION

It has been reported that the erythrocyte membrane contains three different adenosine triphosphatases (ATPases) (Na^+,K^+; Ca^{2+} and Mg^{2+}) (Drickamer, 1975; Ibeh et al, 1992). These ion pumps act in the unidirectional active transport of ions, a process associated with a major role in maintaining the stability of the erythrocyte membrane (Kyte, 1971; Quist and Roufogalis, 1975). There are suggestions that pharmacological agents that alter membrane permeability could be beneficial in the management of sickling of erythrocytes, a major manifestation in sickle cell disease (Dean and Schechter, 1978). Indeed, several reports show that agents that have anti-sickling properties tend to stabilize the erythrocyte membrane (Noguchi and Schechter, 1977; Iwu et al, 1985; Ekeke and Shode, 1990).

In recent times, a number of plant products have gained attention as sources of the active principles in the management of sickle cell disease. Aqueous extracts of Cajanus cajan showed promising returns as antisickling agent (Ekeke and Shode, 1990). Recent studies on the action of aqueous extract of G. kola (a popularly consumed seed in Southern Nigeria, commonly called 'bitter kola') on the osmotic fragility, blood viscosity and reversion of sodium metabisulphite-induced sickling suggested positive effects (Elekwa et al, 2002). The findings support the view that the extract could be beneficial to sicklers.

This report addresses the effect of the G. kola seed extract on the three different membrane ATPases (Na^+, K^+-; Ca^{2+}-; and Mg^{2+}-ATPases) of HbAA, HbAS and HbSS human erythrocytes. The findings would further clarify the possible potentials of the aqueous extract of G. kola in the management of sickle cell disease.

MATERIALS AND METHODS

ATP-Na_2 was purchased from Riedel De Haen, AG Germany, Heparin from Sigma Chemical Co., USA, while other reagents were of the purest grades commercially available.

Blood collection

Sickle cell (HbSS) blood samples were collected from patients who came to the Sickle

I. ELEKWA, Dept. of Biochemistry, School of Biological Sciences, Abia State University, Uturu, Okigwe, Nigeria.
M. O. MONANU, Department of Biochemistry, University of Port Harcourt, P.M.B 5323, Port Harcourt, Nigeria.
E. O. ANOSIKE, Department of Biochemistry, University of Port Harcourt, PMB 5323, Port Harcourt, Nigeria.

Table 1: Activity levels of the different ATPases in erythrocyte membrane preparations from HbAA, HbAS and HbSS blood. Data represent mean ± SD of duplicate determinations.

Genotype	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	Na^+, K^+-ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase
HbAA (n=10)	274.5 ± 2.1	177.6 ± 1.2	103.5 ± 2.3
HbAS (n=10)	256.3 ± 4.5	257.2 ± 0.9	144.2 ± 3.5
HbSS (n=10)	132.6 ± 1.3	293.6 ± 3.1	216.1 ± 1.6

Table 2a: Effect of different concentrations (w/v) of aqueous extract of G. kola seeds on the activity of erythrocyte 'ghost' membrane Na^+, K^+-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of extract (g/ml)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n=5)	HbAS (n=5)	HbSS (n=5)
0.00 (basal)	274.5 ± 2.1	256.3 ± 4.5	132.6 ± 1.3
0.20	310.2 ± 1.5	279.8 ± 3.8	200.3 ± 1.9
0.40	349.8 ± 0.9	310.3 ± 4.1	261.2 ± 2.4
0.80	360.2 ± 2.3	330.1 ± 1.5	270.2 ± 1.5
1.00	360.2 ± 2.3	330.1 ± 1.5	275.7 ± 1.8

Table 2b: Effect of different concentrations (w/v) of aqueous extract of G. kola seeds on the activity of erythrocyte 'ghost' membrane Ca^{2+}-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of extract (g/ml)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n=5)	HbAS (n=5)	HbSS (n=5)
0.00 (basal)	293.6 ± 3.1	257.2 ± 0.9	177.6 ± 1.2
0.20	350.1 ± 1.8	300.1 ± 1.3	229.5 ± 1.4
0.40	389.7 ± 2.3	349.8 ± 2.5	270.1 ± 1.5
0.80	425.1 ± 1.5	380.2 ± 1.7	300.2 ± 2.3
1.00	430.3 ± 0.9	390.1 ± 0.8	305.3 ± 1.7

Cell Clinics of the University of Port Harcourt Teaching Hospital and the University of Nigeria Teaching Hospital at Enugu. Other blood samples came from donors who visited the Medical Centers of the University of Port Harcourt and University of Nigeria, Nsukka Campus. Blood collection was done by veni-puncture into lithium heparinized sterile tubes. The samples were genotyped using standard electrophoretic procedure. Blood samples were stored at 4°C in the refrigerator and used within 24 hours of collection.

Preparation of G. kola seed aqueous extract

The G. kola seeds were purchased from a market at Umuahia, Abia State and were properly identified at the National Root Crops Research Institute, Umudike in Abia State. After peeling the testa, the white seeds were chopped into small pieces and then air-dried. The dried product was homogenized with an electric blender. The coarse product was soaked in distilled water (100 g/ 250 ml) for 24 hours, and the mixture filtered through a clean white cloth. The resultant solution was further passed through Whatman No. 2 filter paper.

Preparation of red cell 'ghosts'

The erythrocyte ghosts from the different genotypes (HbAA, HbAS and HbSS) were prepared according to the method of Hamlyn and Duffy (1978). The whole blood in the heparinized tube was centrifuged at 5, 000 g for 10 minutes. The resultant precipitate was then washed with 0.15 M NaCl, pH 7.4 and re-centrifuged at 5,000 g for 10 minutes. This process was repeated three times. The final precipitate, which contained washed erythrocytes, was lysed by swirling in 5 mM $NaH_2PO_4.2H_2O$ (pH 7.7), and centrifuged at 5,000g for 10 minutes. The resultant precipitate, which contained the pink ghost, was washed with 10 mM Tris-HCl (pH 7.7). The final product was suspended in 3 ml distilled water. The isolated membranes were stored at 4°C and used within 12 hours of collection of blood samples.

Enzyme assays

The assay of the ATPases was performed by the procedure of Hesketh et al (1978), measuring the inorganic phosphate that was released from ATP hydrolysis. The inorganic phosphate was determined using the Fiske and Subbarow (1925) procedure. Enzyme activities were expressed as μmole P_i/mg protein/hour x 10^{-3}.

Na^+, K^+-ATPase: This activity was determined in a reaction mixture containing 0.5 ml each of 0.35 M NaCl, 17.5 mM KCl, 21.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), and 8.0 mM ATP-Na_2. The reaction was initiated by adding 0.2 ml of erythrocyte ghost preparation and incubated at 37°C for 1 hour. The reaction was then terminated by the addition of 0.8 ml of ice-cold 10% (w/v) TCA. The resultant mixture was allowed to stand for 20 minutes at 4°C and then centrifuged at 4, 000 g for 5 minutes. The phosphate concentration was determined in 1 ml of the supernatant by adding 1.0 ml of 2.5% ammonium molybdate. After 10 minutes, 1.0ml of 2% ascorbic acid was added and the mixture kept for 20 minutes at room temperature for colour to develop. The absorbance of the final mixture was then read at 725 nm using SP 6-200 spectrophotometer.

Ca^{2+}-ATPase: The reaction mixture contained 0.5 ml each of 21 mM $MgCl_2$, 17.5 mM $CaCl_2$, 10 mM Tris-HCl buffer (pH 7.4) and 8.0 mM ATP-Na_2. The reaction was initiated by the addition of 0.2 ml erythrocyte ghost preparation and further processed as reported for Na^+ K^+-ATPase assay.

Mg^{2+}-ATPase: The reaction mixture contained 0.5 ml each of 21 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4), and 8.0 mM ATP-Na_2. The reaction was started by adding 0.2 ml of erythrocyte ghost and terminated as described for Na^+, K^+-ATPase assay.

Protein determination

The protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as standard

Kinetic studies of the ATPases

The activities of the different ATPases were determined in the presence of varying concentrations (between 2.0 to 14.0 mM) of substrate, ATP-Na_2. The concentration of inorganic phosphate released was then measured as earlier indicated.

Effect of the aqueous extract on the activity of the various erythrocyte ghosts

The reaction tubes, in addition to the contents reported earlier for the three ATPase assays, contained 0.5 ml of different concentrations (50g/50 ml, 80g/100 ml, 50g/250 ml, and 100g/250ml (w/v)) of the aqueous extract of G. kola seeds. The reaction was then processed as already detailed for Na^+ K^+ ATPase.

RESULTS AND DISCUSSION

The level of activities of the different erythrocyte membrane ATPases investigated in this report are shown in Table 1. The HbSS

membrane showed the lowest Na^+, K^+-ATPase activity but highest Mg^{2+}-ATPase activity, a finding that agrees with the report of Ibeh and Anosike (2000). However, our finding of the highest Ca^{2+}-ATPase activity with HbSS membrane is at variance with that of Ibeh and Anosike (2000), and the reason for this is not certain. Figure 1 shows the double reciprocal plot of the initial velocity data for the three ATPases. Na^+, K^+-ATPase had similar apparent K_m of 5.62+0.02 mM ATP-Na_2 for HbAA, HbAS and HbSS erythrocyte membrane preparations. The apparent maximum velocities ($Vmax_{app}$) were however different. The values expressed in μmole P_i/mg protein/hr x10^{-3} were: HbAA (476.13 ± 1.20), HbAS (454.50 ± 2.50) and HbSS (232.60 ± 1.80). Thus, a lower apparent V_{max} was obtained for the enzyme from HbSS erythrocyte membrane.

The double-reciprocal plot for Ca^{2+}-ATPase activity is shown in Figure 2, which indicates an apparent K_m of the three genotypes as 5.27±0.04 mM ATP-Na_2. The apparent V_{max} values were different with the following values: HbAA (526.30 ± 2.30), HbAS (454.50 ± 6.10) and HbSS (344.80 ± 3.50), in μmole P_i/mg protein/hr x 10^{-3}.

The results for Mg^{2+}-ATPase of the

Table 2c: Effect of different concentrations (w/v) of aqueous extract of G. kola seeds on the activity of erythrocyte 'ghost' membrane Mg^{2+}-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of extract (g/ml)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n=5)	HbAS (n=5)	HbSS (n=5)
0.00 (basal)	103.5 ± 2.3	144.2 ± 3.5	216.1 ± 1.1
0.20	102.3 ± 1.8	143.1 ± 3.4	180.3 ± 1.4
0.40	101.4 ± 2.1	140.5 ± 2.8	129.8 ± 1.7
0.80	100.2 ± 2.4	139.3 ± 1.8	125.1 ± 1.9
1.00	95.1 ± 1.5	139.3 ± 1.8	275.7 ± 1.8

Table 3a: Effect of different concentrations (μM) of phenylalanine on the activity of erythrocyte 'ghost' membrane Na^+, K^+-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of Phe (μM)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n = 5)	HbAS (n = 5)	HbSS (n = 5)
0.0 (basal)	274.5 ± 2.1	256.3 ± 4.5	132.6 ± 1.3
200.0	298.8 ± 1.5	271.2 ± 1.2	185.3 ± 1.1
400.0	331.1 ± 2.39	293.1 ± 1.5	240.1 ± 2.3
600.0	349.8 ± 1.5	315.3 ± 1.3	248.5 ± 0.8
800.0	351.8 ± 1.5	310.3 ± 1.3	249.5 ± 0.5

Phe = phenylalanine

Table 3b: Effect of different concentrations (μM) of phenylalanine on the activity of erythrocyte 'ghost' membrane Ca^{2+}-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of Phe (μM)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n=5)	HbAS (n=5)	HbSS (n=5)
0.0 (basal)	293.6 ± 3.1	257.2 ± 0.9	177.6 ± 1.2
200.0	330.9 ± 1.2	285.4 ± 3.1	220.3 ± 0.6
400.0	395.1 ± 1.7	351.9 ± 1.2	283.1 ± 1.2
600.0	416.3 ±2.3	392.7 ± 0.5	311.6 ± 3.4
800.0	419.1 ± 4.3	398.1 ± 0.5	311.6 ± 3.1

Phe = phenylalanine

Table 3c: Effect of different concentrations (μM) of phenylalanine on the activity of erythrocyte 'ghost' membrane Mg^{2+}-ATPase. Data represent mean ± SD of duplicate determinations.

Concentration of Phe (μM)	Enzyme activity (μmole Pi/mg protein/hour x 10^{-3})		
	HbAA (n=5)	HbAS (n=5)	HbSS (n=5)
0.0 (basal)	103.5 ± 2.3	144.2 ± 3.5	216.1 ± 1.6
200.0	95.3 ± 1.5	139.1 ± 1.5	191.5 ± 0.5
400.0	91.2 ± 1.6	130.6 ± 2.4	132.1 ± 1.9
600.0	85.5 ± 0.6	123.5 ± 1.3	127.8 ± 0.5
800.0	83.9 ± 0.5	123.5 ± 1.3	123.1 ± 1.5

Phe = phenylalanine

different genotypes (Figure 3) show that the apparent K_m for this enzyme was 4.65 ± 0.05 mM ATP-Na_2. In contrast with the values obtained for the apparent V_{max} for the other ATPases studied, for Mg^{2+}-ATPase, the HbSS 'ghost' membrane gave the highest value of 350.90 ± 4.80. The apparent V_{max} values for HbAS 'ghost' was 222.20 ± 5.60 and for HbAA, 187.80 ± 3.45 μmole P_i/mg protein/hr x 10^{-3}. Dixon plots (not shown) were used to determine other kinetic parameters for the effects of the extracts on the different enzymes studied. An activation constant of 0.317 g/ml of extract, for the Ca^{2+}-ATPase of HbSS erythrocyte was obtained while the inhibition constant, K_i, of the extract on Mg^{2+}-ATPase from HbSS erythrocyte by the extract was 1.23 g/ml of extract.

The trend in the variation of Na^+, K^+-ATPase in the three genotypes (HbAA, HbAS and HbSS) observed in this report is very similar to that generally reported by other workers. Thus, a low level of Na^+, K^+-ATPase occurs in HbSS

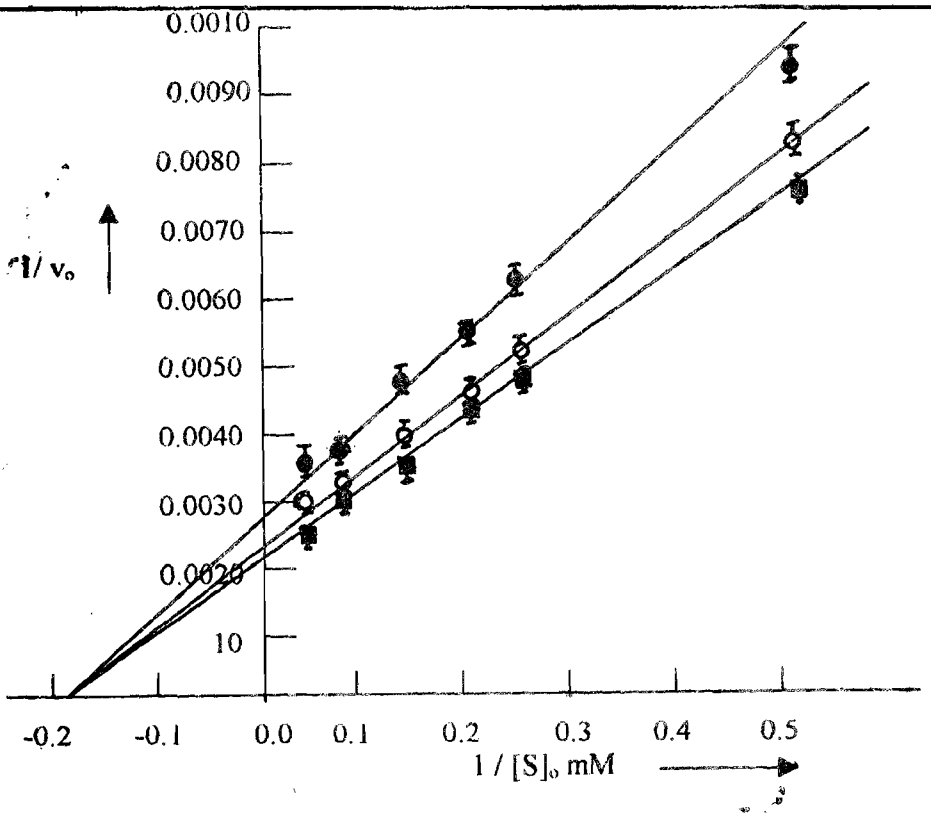

Figure 1: Double-reciprocal plot for Na^+, K^+-ATPase activity from HbAA, HbAS and HbSS erythrocyte 'ghost' membranes (enzyme activity expressed as µmole Pi/mg protein/hour x 10^{-3}). Data represent mean ± SD of duplicate determinations. [■-■, HbAA ; O-O, HbAS; ●-●, HbSS]

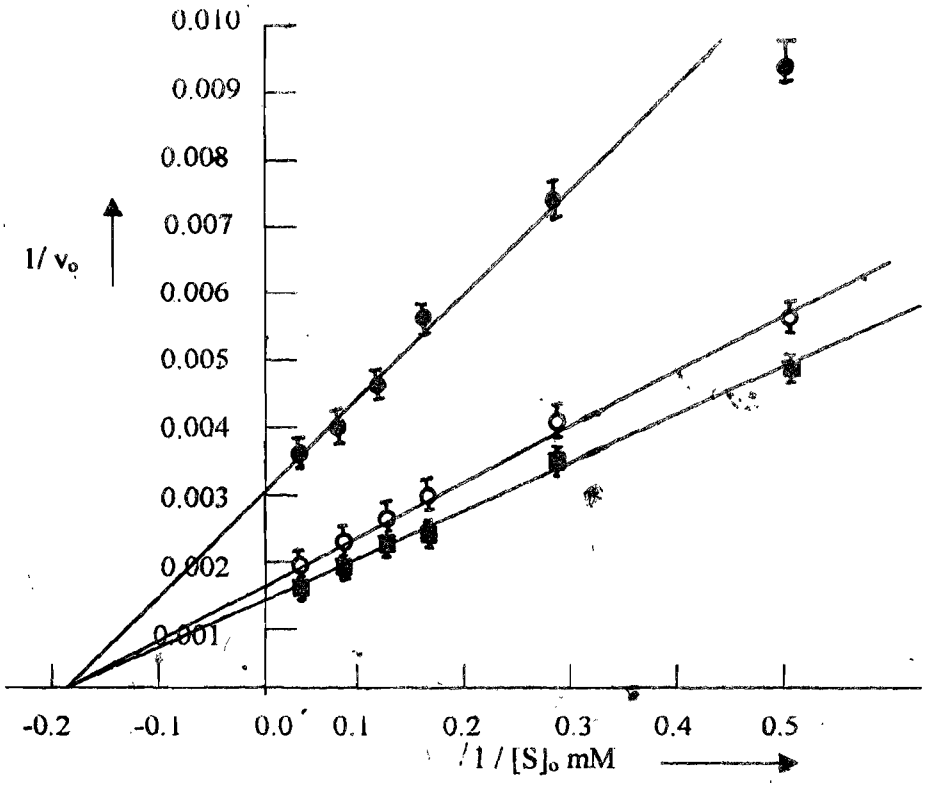

Figure 2: Double-reciprocal plot for Ca^{2+}-ATPase activity from HbAA, HbAS and HbSS erythrocyte 'ghost' membranes (enzyme activity expressed as µmole Pi/mg protein/hour x 10^{-3}). Data represent mean ± SD of duplicate determinations. [■-■, HbAA; O-O, HbAS; ●-●, HbSS]

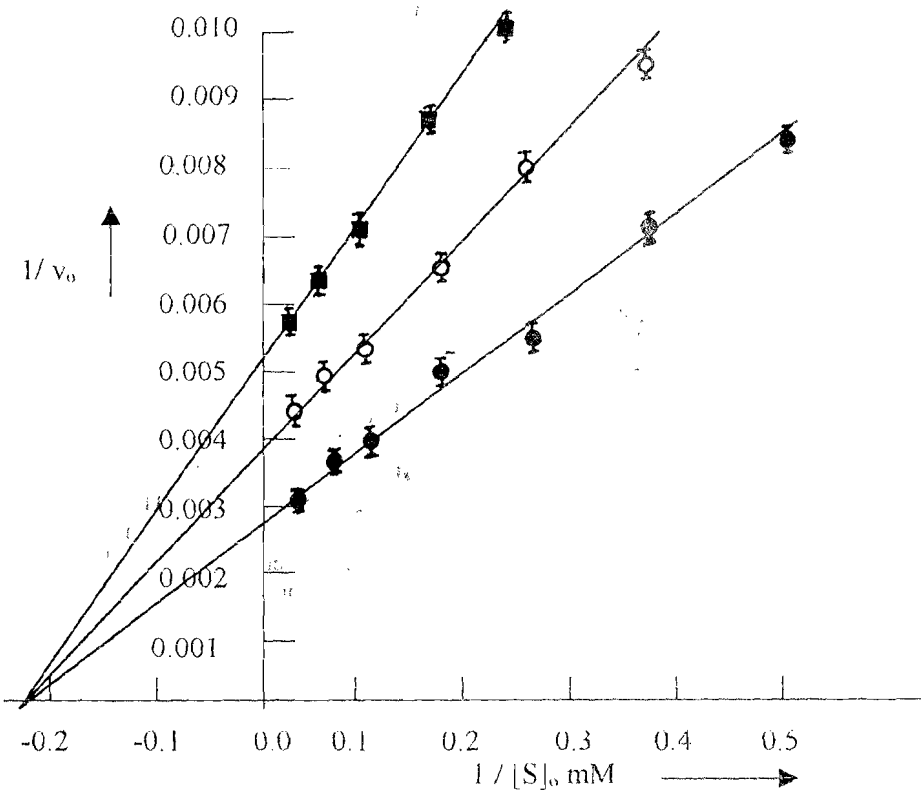

Figure 3: Double-reciprocal plot for Mg^{2+}-ATPase activity from HbAA, HbAS and HbSS erythrocyte 'ghost' membranes (enzyme activity expressed as μmole Pi/mg protein/hour x 10^{-3}). Data represent mean ± SD of duplicate determinations. [■-■, HbAA ; O-O, HbAS; ●-●, HbSS]

erythrocyte membrane when compared with the HbAA erythrocyte (Bewaji et al, 1985; Ibeh et al, 1992). It had been suggested that the intrinsic effect of low intracellular K^+ could account for the low Na^+, K^+-ATPase in HbSS erythrocyte (Friedman et al, 1979), while Kawai and Cowger (1981) attributed the low Na^+, K^+-ATPase of HbSS erythrocyte to an increase in blood bilirubin.

The active extrusion of Ca^{2+} from the cell is ascribed to the membrane-bound Ca^{2+}-ATPase activity. Eaton et al (1973) reported a higher concentration of intracellular Ca^{2+} in HbSS erythrocytes over HbAA erythrocytes, a finding suggested to be due to impaired Ca^{2+}- pump (Bookchin and Lew, 1980). Our findings in this study agree with other reports that show a low Ca^{2+}-ATPase activity in HbSS compared to HbAA erythrocytes (Gopinath and Vincenzi, 1979; Bookchin and Lew, 1980; Ibeh et al 1992). The high Mg^{2+}-ATPase activity of HbSS erythrocyte membrane in comparison with HbAA erythrocyte in this report is in agreement with other studies (Niggli et al, 1982; Bewaji et al, 1985; Ibeh et al, 1992).

The aqueous extract of G. kola seeds, at 0.4 mg/ml had activating effect on Na^+, K^+- and Ca^{2+}-ATPase activities for the three genotypes (Tables 2a and 2b). For Na^+, K^+-ATPase, percentage activations of 27, 21 and 97 were obtained for HbAA, HbAS and HbSS erythrocyte membrane preparations, respectively. The percentage activations for Ca^{2+}-ATPase were 36, 35, and 52 for HbAA, HbAS and HbSS erythrocytes, respectively. Conversely, an inhibitory effect by the extract at 0.4 mg/ml, was observed for Mg^{2+}-ATPase activity (Table 2c). Percentage inhibitions of 2, 3 and 40 were obtained for HbAA, HbAS and HbSS erythrocytes, respectively.

Phenylalanine, a known antisickling agent (Ekeke and Shode, 1990), had an activating effect on Na^+, K^+- and Ca^{2+}-ATPases but inhibited Mg^{2+}-ATPase from the three genotypes (Tables 3a, 3b and 3c). These trends are similar to those observed with the aqueous extract of G. kola seeds. These findings in this report support the view that this extract holds great promise as an antisickling agent, and has potential for the management of sickle cell disease.

ACKNOWLEDGEMENT

The authors gratefully acknowledge financial assistance from the Senate Research Committee, University of Port Harcourt.

REFERENCES

Bewaji, C. O., Olorunsogo, O. O. and Bababumi, E. A., 1985. Sickle cell membrane-bound (Ca^{2+} and Mg^{2+})-ATPase: Activation by 3,4-dihydro 2, 2-dimethyl-2H-1-benzopyran-6-butyric acid. A novel antisickling agent. Cell Calcium 6: 237-244.

Bookchin, R. M. and Lew, V. L., 1980. Progressive inhibition of the Ca^{2+}-pump and Ca^{2+}: Ca^{2+} exchange in sickle red cells. Nature 284: 561-563.

Dean, J. and Schechter, A. N., 1978. Sickle cell anaemia: molecular and cellular basis of therapeutic approaches. New England J. Med. 229: 753-755.

Drickamer, L. K., 1975. The red cell membrane contains three different adenosine triphospnatases. J. Biol. Chem. 250: 1952-1954.

Eaton, J. W., Shelton, T. D., Scoofford, H. S., Kolpin, C. E. and Jacob, H. S., 1973. Elevated erythrocyte calcium in sickle cell disease. Nature 246; 105-108.

Ekeke, G. I. and Shode, F. O. (1990). Phenylalanine is the predominant antisickling agent in Cajanus cajan seed extract. Planta Medica 56: 41-43.

Elekwa, I., Monanu, M. O. and Anosike, E. O., 2002. Effects of aqueous extracts of **Garcinia** kola seeds on membrane stability of HbAA, HbAS and HbSS human erythrocytes. Global J. of Pure and Applied Sciences (in press).

Fiske, C. H. and Subbarow, Y., 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66: 375-400.

Friedman, M. J., Roth, E. F., Nagel, L. R. and Trager, W., 1979. Plasmodium falciparum: Physiological interactions with the human sickle cell. Expt. Parasitol 47: 73-79.

Gopinath, R. M. and Vincenzi, F. F., 1979. (Ca^{2+} + Mg^{2+})-ATPases activity of sickle cell membranes; decreased activation of red blood cell cytoplasmic activator. Am. J. Hematol 7: 303-312.

Hamlyn, J. M. and Duffy, T., 1978. Direct stimulation of human erythrocyte membrane (Na^+-K^+), Mg^{2+}-ATPase activity in vitro by physiological concentrations of d-aldosterone. Biochem. Biophys. Res. Commun. 84: 458-464.

Hesketh, J. E., London, J. B., Reading, H. W. and Glen, A. I., 1978. The effect of lithium treatment on erythrocyte membrane ATPase activities and erythrocyte ion content. Br. J. Clin. Pharmacol. 5: 323-329.

Ibeh, G. O., Anosike, E. O. and Ekeke, G. I., 1992. Effects of age on erythrocyte membrane ATPases in normal and sickle cells. Nig. J. Biochem. 7: 38-46.

Ibeh, G. O. and Anosike, E. O., 2000. Erythrocyte membrane ATPases: effect of hyperphosphataemia on ATPases. Global J. Pure & Appld. Sci. 6: 455-460.

Iwu, M. M., 1985. Antihepatoxic constituents of G. kola seeds. Experientia 41: 699-700.Kawai, K. and Cowger, M. L. (1981). Effect of bilirubin on ATPase activity of human erythrocyte membrane. Res. Comm. Chem. Path. & Pharm. 32: 123-135.

Kyte, J., 1974. Purification of the sodium-potassium dependent adenosine triphosphatase from canine renal medulla. J. Biol. Chem. 246: 4157-4165.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275.

Niggli, V., Adunyah, E. S., Cameron, B. F., Bababumi, E. A. and Carafoli, E., 1982. The Ca^{2+}-pump of sickle cell plasma membranes. Purification and reconstitution of the ATPase enzyme. Cell Calcium 3: 131-151.

Noguchi, C. T. and Schechter, A. N., 1977. Effects of amino acids on gelation kinetics and solubility of sickle haemoglobin. Biochem Biophys. Res. Commun. 74: 637 – 642.

Quist, E. E. and Roufogalis, B. D., 1976. The relationship between changes in viscosity of human erythrocyte membrane suspensions and (Mg^{2+} + Ca^{2+})-ATPase activity. Biochem. Biophys. Res. Comm. 72: 673-679.