Niger. J. Physiol. Sci. 27(June 2012) 035 – 039
www.njps.com.ng

Nig. J. Physiol. Sci.

Assessment and Characterization of Ca^{2+} - ATPase expression in selected isolates and clones of *Plasmodium Falciparum*

***Bolaji O. M., Happi T. C. and Bababunmi E A**
Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria

Summary: Ca^{2+}-ATPase expression in 15 selected isolates from malaria patients at the University College Hospital (UCH) Ibadan and two cloned strains (W2-chloroquine resistant, D6-chloroquine sensitive) of *P.falciparum* was assessed using spectrophotometric assay method. The kinetics of activity of Ca^{2+} - ATPase in three isolates (NCP 14, NCP5, NCP1) and two clones (W2, D6) also assessed. 12% SDS – PAGE analysis of total proteins in one isolate (NCP14) and two clones (W2, D6) was also investigated. All the selected isolates and the two cloned strains exhibited measurable Ca^{2+}-ATPase activity. The Ca^{2+} - ATPase activity in cloned strain D6 (6.50 $\pm$ 0.74µmolPi/min/mg protein) was higher than in cloned strain W2 (3.93 $\pm$ 0.61µmolPi/min/mg protein. The Ca^{2+}-ATPase activity in isolates from malaria patients varied widely (1.95 $\pm$ 0.74 – 21.56 $\pm$1.43µmolPi/min/mg protein). The kinetic constants obtained for the two cloned strains showed that clone W2 had a higher Vmax (Vmax = 363µmolPi/min/mg protein) than clone D6 (Vmax = 74µmolPi/min/mg protein). All the isolates and the two cloned strains showed similar affinity for ATP (Km ~ 10mM). Scan of SDS-PAGE gel of total proteins in the isolate and cloned strains showed the presence of oligopeptide bands of molecular weights range of 148-176 KDa; 116-123 KDa respectively. These suggest the presence of predicted polypeptide of Ca^{2+} - ATPase nature of molecular weight estimate of 139 KDa. The study agrees with previous findings that Ca^{2+}-ATPase is functionally expressed in P.falciparum, The study also indicates that Ca^{2+} - ATPase functional expression may vary with isolate or clone but the ATP binding mechanism to the enzyme is similar in all isolates and clones of *P.falciparum*. The study further suggests a possible association between acquisition of chloroquine resistance and Ca^{2+}- ATPase functional expression in *P.falciparum.*

Keywords*:* *P.Falciparum*, Ca^{2+}-ATPase activity, SDS-PAGE, Chloroquine Sensitivity ATP

**Address for correspondence:* yinkablajim@yahoo.co.uk, +2348023451196
Manuscript Accepted: February, 2012

INTRODUCTION

Growth of the human malaria parasite *P.falciparum* within the red blood cell has been shown to require external calcium ion (Ca^{2+}) and this is associated with a markedly elevated intracellular calcium ion concentration [(Ca^{2+})] (Desai *et al* 1991). The studies of Tanabe et al 1982 has also suggested that invasion of the red cell by plasmodium interferes with the mechanism that regulates intraerythrocytic calcium concentrations. It has been demonstrated that maintaining a high concentration of calcium ion in the Parasitoporous vesicle (PV) is necessary for a normal maturation of P. falciparum within the RBC and that even a short depletion of calcium ion in the PV results in a substantial alteration in the maturation process(Gazarini *et al* 2003)

Ca^{2+}-ATPase in the red cell membranes from schizont-infected erythrocytes reduced by about 30% compared to normal red cells while the calcium content at this stage of infection was between 10 and 20 times that of normal red cells (Tanabe *et al* 1983). The existence of calcium stimulated ATPase in erythrocyte membrane was first demonstrated by Dunham and Glynn (1961) . The enzyme was subsequently shown to transport calcium against a concentration gradient. (Schatzman 1966)

The activities of Ca^{2+}-ATPase in plasma membrane are fundamental to the messenger functions of calcium ions since the enzyme regulates cytosolic calcium ion concentrations (Carafoli 1988). A Ca^{2+}-ATPase gene has been cloned from the genomic libraries of *P.falciparum* and the deduced amino acid sequence of gene product predicted a 139 KDa protein with a total of 1228 amino acids was predicted (Kumura *et al* 1993). The nucleotide and derived amino acid sequence of the ATP-1 gene from *P.falciparum* shares homology with the family of "P"-type cation translocating ATPases in concerned regions important for nucleotide binding, conformations change and phosphorylation (Krishna *et al* 1993).

In this report we examined in vitro, the functional expression of Ca^{2+}-ATPase in 15 selected isolates of

P.falciparum from malaria patients in University College Hospital, Ibadan and two cloned strains (W2, D6).We also attempted characterization of the Ca^{2+}-ATPase expression by studying its kinetic parameters along with SDS-PAGE protein analysis in three isolates and two clones of *P.falciparum.*

MATERIALS AND METHODS

All reagent chemicals were of high grade and products of SIGMA Co. (St. Louis, M O USA). All reagents were filtered prior to use.

Parasites in culture

All the 15 isolates collected from malaria patients from the University College Hospital Ibadan, and the two cloned strains of *P.falciparum* used were maintained in culture according to the procedure of Oduola *et al* 1992.

Free *P.Falciparum preparation* from infected red blood cells.

P. falciparum in culture were harvested at 6-8% parasitemia and centrifuged at 250g at room temperature for 10 minutes. The residue of packed red cells containing the parasites were washed with washing buffer that contained 10mM N-2 Hydroxyethyl piperazine – N-2-ethanesulphoric acid (HEPES), 125mM KCl pH 7.4. The washing was repeated twice and centrifuged at 250g for 10 minutes each time. The final washed residue was suspended in Five (5) volumes of lysing buffer which contained 10mM HEPES, 125Mm kCl, 0.15% saponin pH 7.4 and incubated at 37^0C for 30 minutes. The haemolysate was prepared for ion-exchange chromatography.

Diethylaminoethyl cellulose (DEAE – Cellulose) was equilibrated in washing buffer containing 10mM HEPES, 125mM KCl, pH 7.4 before it was used to pack an elution column. Short elution column was packed and washed with 2-bed volumes of washing buffer. The haemolysate containing *P.falciparum* and ghost red blood cells was loaded on the gel column and eluted by gravity. Collected eluate was centrifuged at 250g for 30 min at room temperature to concentrate isolated *P.falciparum.* The harvested *P.falciparum* was either used immediately or stored for a short term (but used within 24hours) at -20^0C for further studies.

Determination and characterisation of ca^{2+}-atpase activity in isolates of *p.falciparum*

The Ca^{2+}-ATPase activity in the isolates and clones were assayed by a slight modification of Ronner *et al*, (1977).The final concentration of the assay medium contained 120mM kCl, 2mM $MgCl_2$, 0-2mM $CaCl_2$ or 0.5mM EGTA, 30mM HEPES pH 7.4, 10-20µg of isolate of *P.falciparum* in a total volume of 0.8ml. The mixture was pre-incubated for 5 minutes at 37^0C and the reaction was started by adding 1mM ATP, (final concentration). After 30 minutes of incubation at 37^0C with constant shaking, the reaction was terminated with addition of 0.2ml of 5% solution of sodium dodecyl sulphate (SDS) in distilled water. The inorganic phosphate released was determined by a modification of the method of Fiske and Subbarow (1925), ascorbic acid was used as the reducing agent. The colour that developed after 30 min was read at 820nm in a Corning spectrophotometer model 258. Blanks were run to correct for non-enzymic and non-specific hydrolysis of ATP. The, Ca^{2+}-ATPase activity was expressed as the activity in presence of 0.2mM $CaCl_2$ minus activity in presence of 0.5mM EGTA. Each datum represents the average of four independent experimental events. Ca^{2+}-ATPase activity of five isolates NCP 1, NCP 5, NCP 14, D6, and W2 of *P.falciparum* was determined at various concentrations of ATP in order to determine their kinetic parameters.

Determination of protein

Protein concentration in each isolate was determined by the method of Lowry et al (1951) using bovine serum albumin fraction V as standard protein.

Analysis of total protein of isolates of *p.falciparum* on sds-page

SDS-PAGE analyses of cloned strains D6 (chloroquine sensitive) W2 (chloroquine resistant) NCP14 (Isolate from malaria patients at UCH, Ibadan) were performed using the procedure described by Weber and Osborn (1969) to further investigate the expression of Ca^{2+}-ATPase in the parasites. Low and high Mwt standards (26 – 180 KDa were run along with the *P.falciparum* proteins.

The 12% acrylamide gel was stained for 1-2hrs in staining reagent (Brilliant Blue – R250). The gel was destained with several changes of fixing and destaining solution over a period of about 15hrs. Destained gels were scanned on GS300 scanning densitometer. Relative mobility of separated oligopeptides of *P.falciparum* was measured in order to determine their respective molecular weight. The area of each peak was also calculated to determine relative abundance of each oligopeptide to total *P.falciparum* protein applied on the gel.

Statistical analysis

Data obtained were presented as Mean ± SD, comparison of the groups were done using ANOVA (SAS 1987) and student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the summary of Ca^{2+}-ATPase activities in 15 isolates from Nigerian malaria patients and two cloned strains (W2, chloroquine resistant, D6-chloroquine sensitive) of *Plasmodium*

falciparum. All the isolates including the cloned strains exhibited measurable Ca^{2+}-ATPase activity ranging from 1.95± 0.09 to 6.50± 0.74μmolPi/min/mg Protein showed a high level of variability in the species of the parasite.

Table 1. Ca^{2+} -ATPase ACTIVITIES IN SELECTED ISOLATES AND CLONES OF *P. FALCIPARUM*

Sample Identification No.	Specific Activity μmolPi/min/mg Protein, Mean + SD, n=4
D6	6.50+ 0.74
NCP14	5.98+0.08
NCP5	15.24+ 1.69
HF30B	15.15 + 0.92
NCP6	21.56+1.43
NIGIOR	4.67+0.37
W2	3.93+0.61
NCP7	9.93
NIG2OR	6.56+0.32
NL56	1.95+0.09
NCP19	4.31+0.29
JFZ14	6.31+0.27
UNC	2.94+0.37
HFD	8.10+1.89
HFA29D14	1.95+0.74
NCP1	7.62+1.43
MCP13	16.05+0.63

The activity was determined by incubating 10-20μg of *P.falciparum* in assay medium containing 120mM kCl, 2mM $MgCl_2$ 0.2mM $CaCl_2$ or 0.5mM EGTA 30mM HEPES at 37°C for 30mins and measuring the rate of release of inorganic phosphate at γ- position of ATP at 820nm on corning spectrophotometer model 258. Data shown are mean value ±SD from four independent experiments, each performed in triplicate.

Table 2 gives the summary of kinetic values of Ca^{2+}-ATPase activities of three isolates and the two cloned strains of *P.falciparum* studied. Table 3 is the summary of kinetic constants derived from Line weaver – Burk plots of the kinetic values in Table 2.

Table 3 shows that clone W2 (chloroquine resistant) had a higher (fivefold) velocity i.e. potentially faster rate of turnover of product (Vmax = 363μmolPi/min/mg protein) when compared with clone D6 (chloroquine sensitive) with Vmax of 74μmolPi/min/mg protein. The Vmax for the three isolates from Nigerian patients were higher than that of clone D6 but lower than that of clone W2. All the isolates and the two cloned (W2, D6) strains showed similar affinity for ATP (Km ≃ 10.0mM.)

Fig 1 is the scan of SDS-PAGE gel of total proteins in three isolates of *P.falciparum.* Table 4 represents the analysis derived from the scanned gel. Fig. 1 The Table shows that each of the isolates contained six (6) major oligopeptide bands, indicating that the

Table 3. Summary of kinetic values of the Ca^{2+} -ATPase activity in five selected isolates of *p. falciparum* characterised.

Sample Identification	**Km (mM)**	**Vmaxμmolpi/min/mg Protein**
NCP14	11.0	99.0
NCP5	9.0	172.0
NCP1	7.5	140.0
W2	10.0	372.0
D6	10.0	74.0

The values were derived from Line weaver – Burk plots of values in Table 2. Km=Affinity of Protein for ATP, Vmax=Maximum velocity of activity of the Protein

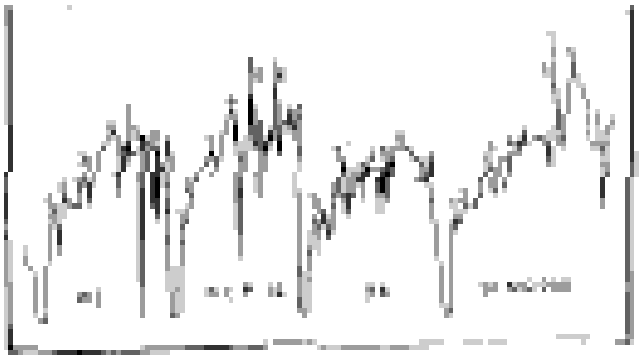

Figure 1. Scan of SDS-page of total proteins in one isolate and two clone strains of *p.falciparum.* Proteins were denatured by solubilising in sample buffer that contained 25mM Tris, 4% SDS, 20% Glycerol 10% mercapto-ethanol pH 6.8. The electrophoresis was run on 12% acrylamide slab gel at constant current of 30mA. After the run, the gel was stained for 1-2hrs in staining reagent (Brilliant Blue – R250). The gel was destained with several changes of destaining solution over a period of 15hrs. Destained gel was scanned on G5 300 scanning densitometer. Relative mobility (R_f) of separated oligopeptides in samples were measured and the area of each peak was also calculated.

Table 2. Kinetic studies of Ca^{2+}- ATPase activity of some isolates of *p. falciparum*

[S] ATP mM	**$[^1/_S]$**	**μmolPi/min/mg Protein**					**$[^1/_V]$ x 10^{-2}**				
		NCP1	NCP 5	NCP14	W2	D6	NCP1	NCP 5	NCP14	W2	D6
5.0	0.20	45.03	33.03	17.78	82.90	15.07	2.22	3.03	5.62	1.21	6.64
10.0	0.10	97.41	86.98	40.54	184.46	39.65	1.03	1.15	2.48	0.54	2.52
15.0	0.07	139.95	176.79	65.36	280.81	61.10	0.71	0.71	1.53	0.36	1.64
20.0	0.05	142.18	175.80	93.06	363.72	74.13	0.70	0.70	0.07	0.27	1.35
25.0	0.04	52.38	53.77	97.05	334.20	35.98	1.91	1.91	1.03	0.30	2.78

The kinetics was determined by measuring the rate of release of inorganic phosphate at various concentrations of ATP, as described in Table 1. S=Substrate ATP concentration, V=Specific activity of Ca^{2+}-ATPase (μmolpi/min/mg Protein), I/(S)=Reciprocal of substrate concentration, I/V=Reciprocal of specific activity

Table 4. Analysis of SDS-PAGE of total proteins of selected isolates and cloned strains of *p. falciparum*

Bands	KDa MW Ranges	% W2	% NDP 14	% D6
I	148 – 176	4.6	7.7	10.9
II	116 – 123	7.4	11.0	12.0
III	67 – 80	7.8	28.5	34.8
IV	37 – 48	21.2	24.4	21.4
V	25 – 34	7.0	21.1	10.7
VI	19 – 36	51.9	7.3	5.2

Relative mobility (Rf) of standard Mwt proteins (26-180Kda) and separated oligopeptides from samples were measured. A graph of R_f Vs Mwt of standard proteins was plotted. The molecular weight of each separated oligopeptide in *P.falciparum* was extrapolated from the graph. Area of each peak corresponding to each oligopeptide was calculated relative to total area of protein applied onto the gel. Data shows the molecular weight estimates and relative abundance of each oligopeptide as a percentage (%) of total protein applied.

oligopeptides in the isolates had similar mobility pattern as a result of identical or similarity in the constitution of the oligopeptides. The relative quantities of oligopeptides in each band (expressed as a percentage of total protein applied) indicate that there were variations in the quantity of the oligopeptides in each band of the isolates; thus suggesting probable existence of polymorphism in protein synthesis in *P.falciparum*.

The presence of prominent bands (Bands I & II) in Table 2, with molecular weights range 148-176 KDa and 116-123 KDa respectively suggest the presence of predicted oligopeptide of Ca^{2+}-ATPase nature of molecular weight estimate of 139Kda in the parasite candidates.

DISCUSSION

ATP-dependent Ca^{2+}-ATPase has been reported in a variety of plasma membranes where they serve to maintain a 10,000 fold calcium ion gradient across the membranes. (Sarkadi1980; Schartzmann 1982).

Its operation is critical both to cell survival and to the role of Ca^{2+} as a second messenger which mediates the coupling of surface excitation to cellular responses (Carafoli 1984, Rasmussen 1970)

We reports the presence of functional Ca^{2+}-ATPase activity in 15 selected isolates from malaria patients and two cloned strains of P.falciparum. The assayed ATPase activity is in agreement with the evidence of Kumura *et al* (1993) who cloned a Ca^{2+}-ATPase gene from the genomic library of *P.falciparum* that the parasites contain the enzyme. The deduced amino acid sequence of the product from the gene cloned by them indicated a 139KDa protein with a total of 1228 amino acids. Our study showed variations in the functional expression of Ca^{2+}-ATPase in isolates and clones of P. falciparum.Variations in the magnitude of the Ca^{2+}-ATPase activities in the isolates and the clones shown in this study might signify differences in the expression of the enzyme in P.falciparum generally.

The kinetic constants derived from the kinetic studies of the Ca^{2+}-ATPase in 3 randomly selected isolates and two clones of *P.falciparum* showed a high Km of 10mM thus indicating low affinity for ATP. Ca^{2+}-ATPase of the plasma membrane of eukaryotic cells interacts with calcium with high affinity (Km of about 0.5μmol) but has low total transport capacity (Carafoli 1988). The high calcium affinity makes it able to export calcium from cells continuously, thus making it play the most important role in maintaining the 10,000 fold gradient of calcium between cells and its environment.(Carafoli 1988) Also, the reconstituted Ca^{2+}-ATPase in human erythrocyte showed that the protein transports calcium with 1.1 stoichiometry to ATP hydrolysis (Carafoli 1988). We suggest that the calcium transport capacity of Ca^{2+}-ATPase in *P.falciparum* may be similar to those of other eukaryotic cells. The purified enzyme in eukaryotic cells indicate that it is a single polypeptide of about 138KDa and an ATPase of the P-class i.e. form an aspartyl phosphate during its reaction with ATP (Carafoli 1988). The result of the densitometric scan of SDS-PAGE of total protein of *P.falciparum* showed the presence of polypeptide in bands I and II with molecular weights range 148-176 KDa and 116 – 123 KDa respectively. Though we did not identify a specific protein band with molecular weight of 139KDa, we suspect that this protein may be inclusive in band 1region of the scan.

Kinetics of Ca^{2+}-ATPase activity in *P.falciparum* showed that clone W2 (chloroquine resistant) had a higher potential of (fivefold) rate of turn over of product (hydrolysis of ATP) (Vmax = 363μmolPi/min/mg protein) when compared with clone D6 (chloroquine sensitive) with Vmax of 74μmolPi/min/mg protein. The rate of hydrolysis of ATP for the three isolates from Nigerian patients at the University College Hospital, Ibadan was higher than that of clone D6 but lower than that in clone W2.This result may suggest variations in kinetic characteristics of the enzyme in different isolates and clones of P. falciparum. The reconstituted enzyme from human erythrocyte in previous studies showed that the enzyme

transports calcium with 1:1 stoichiometry to ATP hydrolysis (Carafoli 1988). It can then be deduced that clone W2 has a higher potential for calcium ion transport across the membranes than clone D6. The study may therefore suggest a possible association between Ca^{2+}-ATPase functional expression and drug resistance in *P.falciparium.* All the isolates including the cloned strains showed similar affinity for ATP (km $\underline{\sim}$ 10mM) indicating that ATP binding mechanism to the enzyme is similar in all isolates and clones of *P.falciparum* and it is independent of chloroquine sensitivity of the parasite. Specificity and sensitivity of thapsigargin have been tested on several types of endoplasmic reticulum Ca^{2+}-ATPase family (SERCA) and have been shown that thapsigargin inhibited all of the SERCA isozymes with equal potency (Carafoli 1988, Lytton *et al* 1991). It has been suggested that Ca2+-ATPase cloned from genomic library of P falciparum has amino acid sequence homology of 45-50% to vertebrate sarcoplasmic reticulum(SERCA) Ca^{2+}-ATPase(Carafoli 1988). Also artemisinin (an antimalarial) has been shown to inhibit the SERCA orthologue (PfATP6) of plasmodium falciparum expressed in *xenopus oocytes* with similar potency to thapsigargin thus it might be explained that one of the mechanisms of action of artemisinin during chemotherapy is via inhibition of Ca^{2+}-ATPase activity (Eckstein-Ludwig *et al* 2003, Dahlstrom *et al* 2008). Our study was unable to localize the identified Ca^{2+}-ATPase. This is a subject for further study.

Acknowledgement
We thank WHO/TDR who provided the fund for this study.

REFERENCES

Carafoli E. (1984). Plasma membrane calcium transport and Ca^{2+} binding by intracellular stores: an integrated picture with emphasis on regulation. In: Mechanism of Intestinal Electrolyte transport and Regulation. Alan R. Liss N.Y pp 120-134

Carafoli E. (1988). Intracellular calcium regulation with special attention to the role of the plasma membrane calcium pump. J. Cardio Pharm. 12; 577-584

Dahlstrom S, Veiga M I, Ferreira P, Martensson A, Kaneko A, Andersson P, Bjorkman A, Gil JB (2008).Diversity of the sarco/endoplasmic reticulum Ca2+-ATPase orthologue of P. falciparum (PfATP6). Infect. Genetic Evo.8(3): 340-345

Desai S.A., McLeskey E.W., Schlesinger P.H., Krogstad D.J. (1996). A novel pathway for calcium ion entry into *Plasmodium falciparum* infected blood cells. Amer. J. Trop. Med. 54(5); 464-470

Dunham E.T and Glynn I.M (1961). Adenosine triphosphate activity and the active movements of alkali metal ions. J. Physiol. (Lond.) 156; 274-293

Eckstein-Ludwig U, Webb R J, van Goethem I D A, East J M, Lee AG, Kimura M, O Neill P M, Bray P G, Ward S A and Krishna S (2003). Artemisinins target the SERCA of Plasmodium falciparum. Nature 424; 957-961.

Fiske C.H. and Subbarow Y (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66; 375-400

GazariniML,Thomas A P,Pozzan T, Garcia CRS (2003).Calcium signaling in a low calcium environment: how the intracellular malaria parasite solves the problem Cell Biol. 161(1):103-110

Krishna, S., Cowan G., Meade J.C., Wells R.A., Stringer J.R., Robson K.J. (1993). A family of cation ATPase-like molecules from *P.falciparium* J.Cell Biol. 120(2); 385-398.

Kumura M., Yamaguchi Y. Takada S, Tanabe K. (1993). Cloning of Ca^{2+}-ATPase gene of *P.falciparum* and comparison with vertebrate Ca^{2+}-ATPase. J. Cell. Sci. 104 (4); 1129-1136

Lowry O.H., Rosebrough N.J., Farr A.L. and Randal R.J. (1951). Protein measurement with the Folin phenol reagents. J. Biol. Chem. 193; 265-275.

Lytton J, Westlin M and Hanley M R (1991). Thapsigargin inhibits the sarcoplasmic reticulum Ca2+-ATPase family of calcium pumps. J. Biol. Chem. 266(26); 17067-17071.

Oduola A.M.J., Ogundahunsi A.T., Salako L.A. (1992) Continuous cultivation and drug susceptibility testing of *Plasmodium falciparum* in a malaria endemic area. Protozool. 39(5); 605-609.

Rasmussen H. (1970) Cell communication, calcium ion and cyclic adenosine monophosphate – Science 170; 405-412

Ronner P, Gazzotti P, and Carafoli E, (1977). A lipid requirement for the Ca^{2+}, Mg^{2+} -ATPase of erythrocyte membranes. Archs. Biochem Biophys. 179; 578-583

Sarkadi B (1980). Active calcium transport in human red cells. Bioclin. Biophys. Acta 604; 159-190

Schaztman H.J. (1966). ATP dependent calcium ion extrusion from human red cells. Experentia 22; 364-365.

Schartzmann H.J.(1982) The plasma membrane calcium pump of erythrocytes and other animal cells. In: Membrane Transport of Calcium. Carafoli E. (ed). Academic Press London pp 40 – 108

Tanabe K, Mikkelsen R.B. and Wallach D.F. (1982). Calcium transport of *Plasmodium chabaudi* infected erythrocyte. J. Cell Biol. 93; 680-684

Tanabe K, Mikkelsen R.B. and Wallach D.F. (1983). In: Malaria and the red blood cell (Everad and Whelhan J. Eds.) CIBA Foundation symposium Pitman pp 64-73

Weber K, and Osborn M (1969). The reliability of molecular weight determination by dodecyl sulphate polyacrylamide gel electrophoresis. J. Biol. Chem. 244 4406-4412.