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Asymmetry of the Malonate Transport System in Human Red Blood Cells

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Abstract

Transport kinetics of [2-^{14}C] malonate influx and efflux by the human erythrocyte membrane have been determined as $K_{m(cis)}$, 21mM and $K_{m(trans)}$, 3.2 mM.The flux of this organic dianion is asymmetrically concentration, pH and temperature dependent. Competitive inhibition of malonate by SITS, BPS and sulphate strongly implies that a common route exists for both inorganic anions and organic dianions, namely the anion-exchange band 3 protein. ^{14}C-Malonate which is nonmetabolized in the erythrocyte, is a useful kinetics probe for monitoring membrane potential and self- inhibition.

Keywords: Malonate transport erythrocytes; band 3
Abbreviations: SITS, 4-Acetamido-4'-isothiocyano-2, 2'-disulfonicstilbene; BPS, Bathophenanthroline disulphonate; cis: Outward-facing; trans: Inward-facing; Hct: Haematocrit

Introduction

The anion exchanger-1 AE1 (synonymous with Band 3; capnophorin; gene SLC4A1) is the most abundant human red cell membrane protein. It is part of a family of anion exchange proteins found in the plasma membrane of various cell types (Passow, 1986; Salhany, 1990; Jennings, 1992; Tanner, 1993; Reimeier, 1993; Gibson and Ellory, 2002). AE1 is consistent with its classical function, electroneutral HCO_3- - Cl- exchange which has to be achieved in the 300-600ms period for which the red cell traverses the pulmonary circulation and hence, a high rate of anion flux is necessary (Salhany,1996; Ellory et al, 2009). These transporters can also transport a wide variety of other anions (Jenning, 1992). Certain members of the anion exchange transporter family also have the secondary function of providing cytoskeletal and cytosolic protein binding sites at the inner surface of the membrane in which the latter function is primarily assigned to an N-terminal cytoplasmic extension (Low, 1986; Salhany, 1990; Jennings, 2005). In recent years, our understanding of the anion exchange system of the human red blood cell has been greatly augmented by two sorts of information. On the one hand, kinetic studies have shown that the transport displays properties consistent with a mobile carrier model (Knauf et al., 2002). On the other hand, the use of the inhibitory chemical probes have

lead to the identification of the 95,000-dalton polypeptide (Fairbanks et al., 1971).Band 3 makes up ~25% of the total red cell membrane protein and is present in the membrane as ~550,000 noncovalent dimmer (Verkman et al., 1983). To integrate these two kinds of information, additional knowledge is required concerning the relationship between carrier sites postulated from kinetic analysis of transport and inhibitory sites in band 3 by the covalent reactions of chemical probes. Although the majority of studies associated with this protein have been centred on inorganic anion (Fievet et al., 1998), organic anions are kinetically more informative (Hajjawi and Hider, 1987). The evaluation of the mechanism by which probes inhibit anion transport can be greatly facilitated by the use of "bimodal" membrane topology inhibitors (Reithmeier, 1993). Although Wood et al.(1992) reported that a mutation of two lysines were involved in the covalent binding of 4,4'-diisothiocyanodihidrostilbene-2,2'-disulfonate (H_2DIDS), but kinetic studies have shown that lysine does not participate in the substrate binding site (Passow,1986). Also, Okubo et al. (1994) lowered the affinity of 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) by two orders of magnitude without altering the chloride binding K_m significantly. While these results may suggest that the inhibitor and substrate binding sites differ, it is possible that there is only one substrate binding site in the native state and that proteolysis or mutation leads to selective blockade of access to the transport site for stilbenesulfonates, but not for chloride. Hajjawi and Hider (1987) have reported that the rate of maleate (*cis*, intercharge distance 3.7 Å) uptake is markedly greater than fumarate (*trans*, intercharge distance 5.7 Å). This specificity of dicarboxylate uptake by human erythrocytes is qualitatively similar to that reported for bovine erythrocytes (Passow, 1986). Aubert and Motais (1975) proposed a "three point attachment" hypothesis for hydrophilic anions and it was concluded that a selectivity filter of diameter 7.6-9.0 Å was a major determinant of permeability. Significantly, all the dicarboxylates capable of rapid permeation of human erythrocyte membranes are planar, and in view of their small intercharge distance, are capable of forming tight bidentate hydrogen bonded ion pairs with a side chain of "arginine" (Hajjawi and Hider, 1987). Similar complexes are formed between both sulphate, phosphate and argentine and such interactions are quite stable in aqueous solutions (Tatham et al., 1983). Zaki (1990) has argued for the presence of 2 to 3 arginine residues at AE1. Objective tests of the mechanism of competition within the native state of the transporter require studying the effect of malonate on the kinetics of stilbenedisulfonates binding (Jennings, 2005). To answer outstanding questions about the mechanism of competition, we began extensive quantitative analysis of the kinetics of SITS and BPS binding sites to band 3. SITS and BPS which are nonpenetrating, very specific anion transport inhibitors that allow the nature of their effects to be determined by kinetic analysis.

The present paper is concerned with providing more information about the specificity and kinetic parameters of the anion transporter at both outside and the inner side of human erythrocytes. We determined K_m, K_i and pH_{max} values for the effect of organic and inorganic anions on malonate influx $_{zero\text{-}trans}$ and efflux $_{zero\text{-}cis}$. We also report on the effect of pH on membrane potential value of malonate distribution ratio for free-energy differences between the various conformations of human AE1. This work has been pursuing the asymmetry flux of malonate, anion homoexchange, to provide means of determining what changes in molecular structure that is more likely to take place during the transport process ($E_i \leftrightarrow E_o$) in AE1.

Materials and Methods

[2-^{14}C]Malonate and [U-^{3}H]inulin were purchased from Radiochemical Centre, Amersham, Buck, UK; Silicon oil was purchased from Hopkin and Williams, Romford, Essex, UK. All other materials were supplied by Sigma (London) Chemical Co., Poole, Doreset, UK, or by BDH Ltd., Poole, Dorset, UK, and were of AnalaR grade whenever possible. All solutions were prepared with glass-distilled water.

Erythrocyte Preparation

Freshly outdated blood containing citric acid, sodium citrate, dextrose and adenosine was collected from Essex Country Hospital, Colchester, UK. Human erythrocytes were separated by sedimentation at 2500 g (at r_{av} 13 cm) and 4ºC for 5 min using MSE Mistral 4L. The plasma and buffy coat were removed by aspiration. The erythrocytes were then washed three times by resuspension and sedimentation in ice-cold NaCl (152 mM). Finally, erythrocytes were suspended in Krebs-Ringer bicarbonate (Umbreit et al., 1946) at pH7.4, 1:1 (v/v) for isotope flux studies, and kept on ice. Erythrocytes were always used for experiments immediately after preparation.

The volume of erythrocyte intracellular water (68.8%) was determined with the use of [^{3}H]inulin as described by Winter and Christensen (1964).The standard dry weight of erythrocyte suspension (typically ~30%) was used to calculate the intracellular fluid for each experiment.

[2-^{14}C]Malonate Flux Studies

[^{14}C]Malonate influx was measured using a slight modification of the procedure reported by Eavenson and Christensen (1967) for pigeon erythrocytes. An isoosmotic malonate/sucrose solution (1ml, pH7.4) containing 0.05-0.25 μCi/ml was pipetted into a test tube and preincubated at 37 ºC for 15 min. An erythrocyte suspension (packed cells: Krebs Ringer bicarbonate buffer, 1:1 by vol, 1 ml) was then added, oxygenated and incubated. The media was shaken mechanically throughout the incubation period. At predetermined intervals 0.5 ml of incubation medium was added to polypropylene conical tubes (1.5 ml) containing 0.5 ml of silicon oil (ρ 1.07) and centrifuged for 20 s in a Beckmann Microfuge B (Rosenberg and Rafaelsen, 1979). Aliquots of the supernatant fluid (0.1 ml) were taken for scintillation counting, the remainder being removed by aspiration. Extra care was taken not to aspirate portions of the pellet while removing the oil. H_2O (0.25 ml) was then added to the packed cells with vigorous mixing. The resulting haemolyzed erythrocytes were bleached with 10% trichloroacetic acid (0.25 ml). The denatured material was sedimented by centrifugation and a 0.1 ml sample of supernatant fluid was taken for scintillation counting.

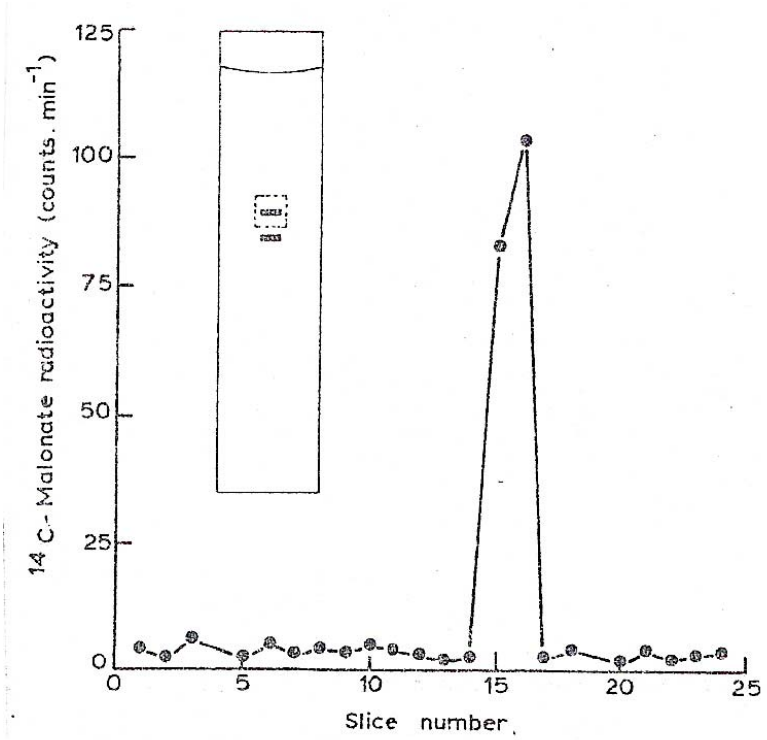
Under these incubation conditions, with extracellular chloride ~ 40mM, chloride ions efflux from the cell at the onset of the incubation due to the chloride concentration gradient across the erythrocyte membrane. This net flux creates a diffusion potential which is positive inside. This diffusion potential increases both the initial rate and the distribution ratio of malonate (unpublished observations). These incubation conditions were used throughout the study. Similar results were obtained by substituting Krebs Ringer bicarbonate buffer by phosphate/citrate, Tris-HCl, Sorenson's phosphate or glycine/NaOH buffers (pH 7.4). During the initial phase of uptake experiments, the influx of malonate in human erythrocytes is effectively irreversible and is pseudo-zero order (Michaelis and Menten, 1913). The rate of influx of malonate anions (1 to 20 mM) was linear for time period up to 5 min. Fixed time assays were run either for 1 min or 2 min intervals. Values are presented as mean ± SD, based on 3 measurements per sample.

It is assumed that the properties of the [^{14}C]malonate tracer do not differ from those of the nonradioactive [^{12}C]malonate.

Results

The Metabolism of [^{14}C]malonate dicarboxylates in the cytoplasm was investigated by virtue of incubating erythrocytes (25% Hct) in isoosmotic malonate solution (5 mM) at 37 ºC and the influx of $malonate_{zero\ trans}$ was allowed to reach equilibrium ($r = [Malonate]_i / [Malonate]_o = 1$). A sample of intracellular fluid was administered to a thin layer chromatography (TLC) sheet (). Malonate dissolves in ethanol, thus the developing solvent was absolute ethanol/water (60:40 (v/v)).The TLC was divided into fractions which were counted for radioactivity (Fig. 1).

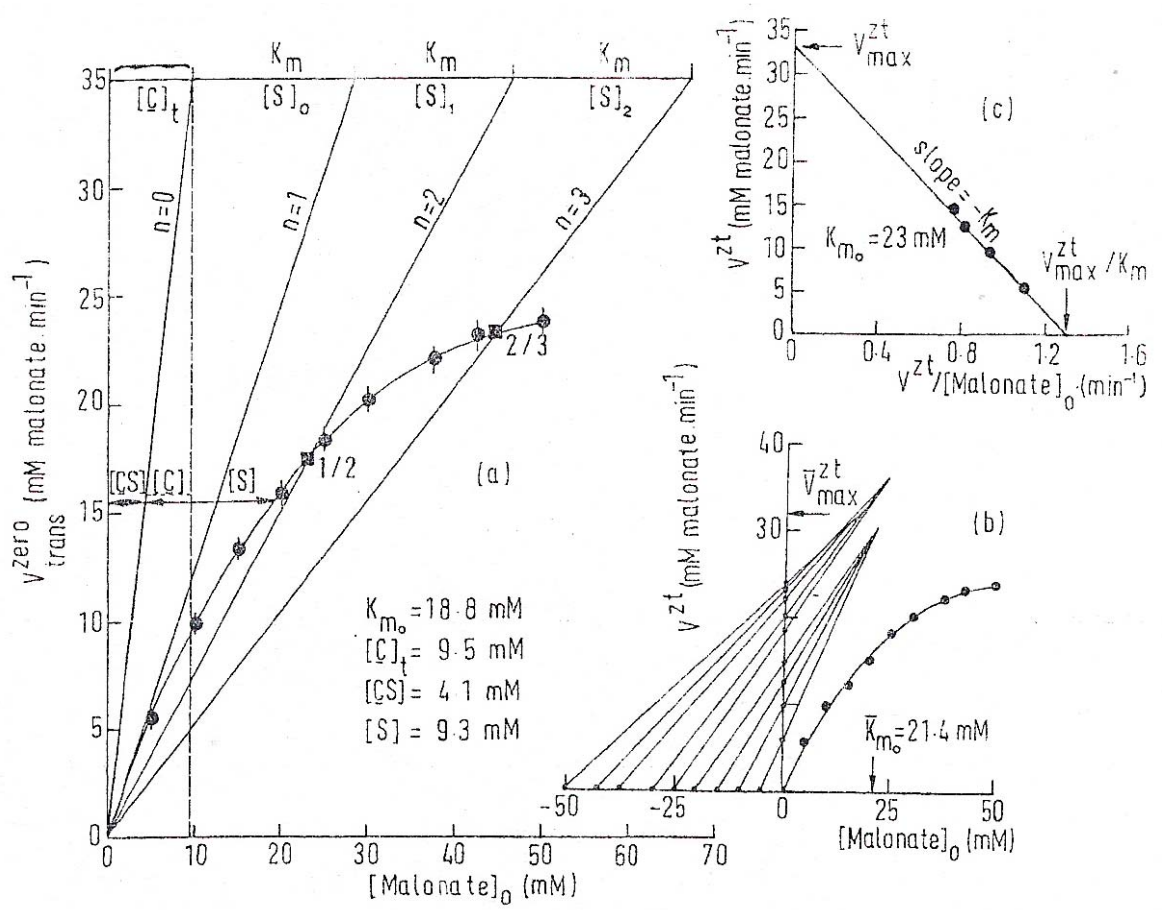
Figure 1: Metabolism of [^{14}C]malonate.
For details see the text. A plot of ^{14}C-radioactivity vs. fraction number of thin layer chromatography. Inset: Intracellular fluid was administered to silica gel (60 Å and F_{254nm}) which was developed in absolute ethanol: water, 60:40 (v/v).The boxed slice corresponds to the peak of ^{14}C-malonate.



The distance moved by malonate (4.7 cm) in the direction of developing solvent flow (7.8 cm) during a chromatographic separation is characterized by the term R_F value, which was 0.6. The slightly slower running spot (R_F = 0.54) was associated with an endogenous iodine-positive compound. There was no detectable radioactivity associated with this compound. It is therefore clear that no appreciable metabolism of malonate had occurred.

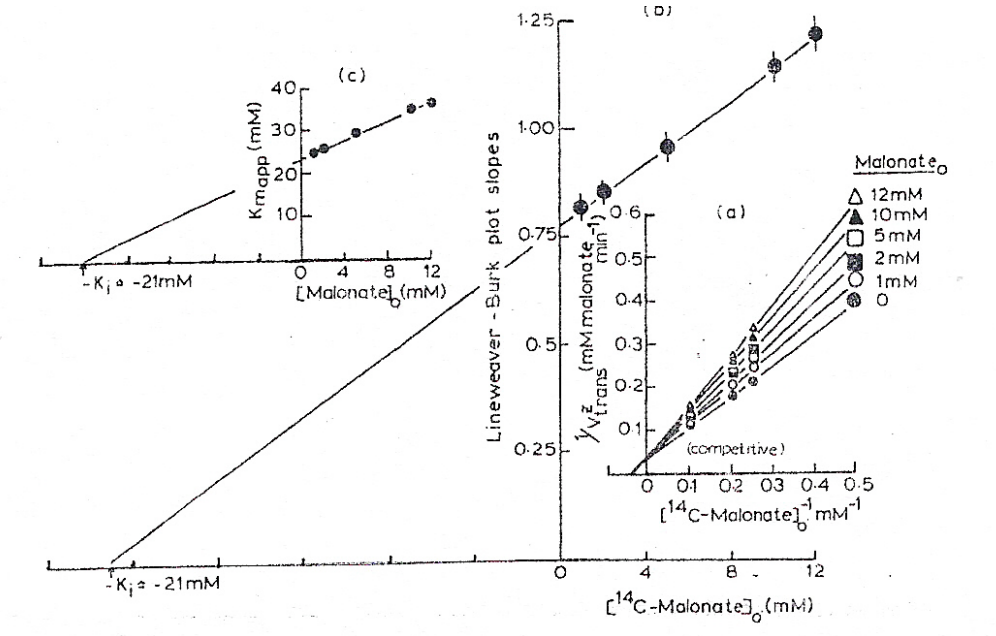
Since malonate has been established to be nonmetabolized in human erythrocytes, and it permeates these cells rapidly. As a result it was selected as a probe for isotope uptake studies. The concentration of malonate was limited to the range 1-20 mM, because under such conditions the trans membrane gradient of malonate failed to change the biconcave shape of erythrocytes as demonstrated by light scattering studies and 'dry weight' measurements (Hajjawi and Hider, 1987). Influx was analysed in terms of Michaelis-Menten kinetics. Under the adopted incubation conditions, the kinetic parameter ($_{Km(cis)}$) for malonate influx is an appearant value, accommodating the inhibitory influence of chloride. However, as the extracellular chloride concentration (40 mM) was maintained throughout the entire range of experiments, the appearant kinetic constants are useful in the analysis of comparative inhibitory potencies of anions. Using Dixon, Eisenthal-Cornish-Bowden and Wolf-Hofstee plots a K_m value of 21 mM was obtained (Fig. 2). The Lineweaver-Burk plots were consistent throughout the malonate uptake studies (Hajjawi and Hider, 1987).

Figure 2: The Michaelis Constant for [^{14}C]malonate uptake
For details see the text. (a) Dixon plot; (b) Eisenthal-Cornish-Bowden plot; (c) Woolf-Hofstee plot



The rate of [^{14}C]malonate uptake was decreased at the increasing level of [^{12}C]malonate (Fig. 3). The self-inhibition, K_i, was shown to be 21mM.

Figure 3: Effect of [^{12}C]malonate [$_{cis}$] n [^{14}C]malonate uptake.
Erythrocytes (25% Hct.) were incubated in isoosmotic [^{14}C]malonate Solution, [S], containing constant concentrations of [^{12}C]malonate, [I],(pH 7.4), self-inhibition. Total malonate concentrations were limited such that there was no detectable lysis. (a) Lineweaver-Burk plots for [^{14}C]malonate influx vs. medium substrate concentrations as afunction of malonate concentrations: ●, control; ○, 1 mM; ■, 2 mM;□, 5mM; ▲, 10 mM; Δ, 12 mM. (b) Slopes of Lineweaver-Burkplots vs. $[S]_o$. (c) Apparent K_m's of Lineweaver-Burk plots vs. $[S]_o$.



The uptake of [^{14}C]malonate was further examined by measuring the inhibitory effect of both BPS (Fig. 4) and SITS (Fig. 5) which are known to bind the external portion of the anion exchange band 3 protein (Zaki,1990) at K_i value of 0.7mM and 1mM, respectively. The inhibitory effect of other mono-, di- and tricarboxylic acids and inorganic anions were reported by Hajjawi and Hider (1987).

Figure 4: Effect of BPS on [^{14}C]malonate uptake
Erythrocytes (25% Hct.) were incubated at 37 oC in isoosmotic [^{14}C]malonate solution pH 7.4) which contain a fixed concentration of BPS molecules. (a_1) The rate of zero-trans influx of [^{14}C]malonate was assayed as a function of medium BPS concentration, ●, control; ○, 0.25 mM; □, 0.5mM; Δ, 1.0 mM (a_2) the slopes of Lineweaver-Burk plots vs. medium concentration of BPS. (a_3) Erythrocytes (25% Hct.) was loaded with [^{14}C]malonate solution (12 mM, pH 7.4) at 37ºC for 40 min. The incubation was quenched on an ice-bath and the medium centrifuged (r_{av} 13 cm) at 4ºC for 5 min. The supernatant was aspirated and the packed cells washed (by suspension, centrifugation and aspiration) three times in NaCl (150 mM).The cells were then incubated in isoosmotic sucrose containing 1mM BPS at 37ºC. ▲, efflux of [^{14}C]malonate in the presence of BPS_o; Δ, cells were pre-incubated in BPSo (1mM) for 40 min and medium BPS was washed away (by suspension, centrifugation and aspiration) three times in KRB buffer, and then incubated in BPS-free medium; □, efflux of [^{14}C]malonate in the absence of BPS.

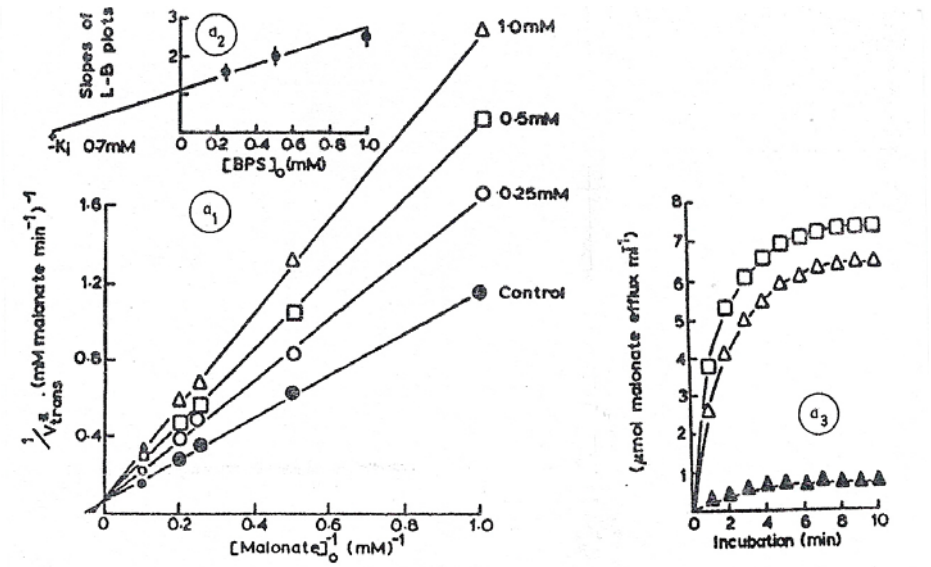
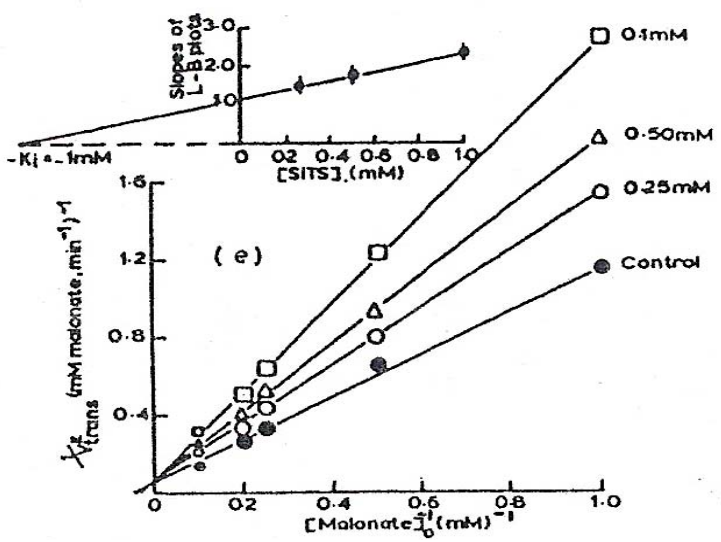
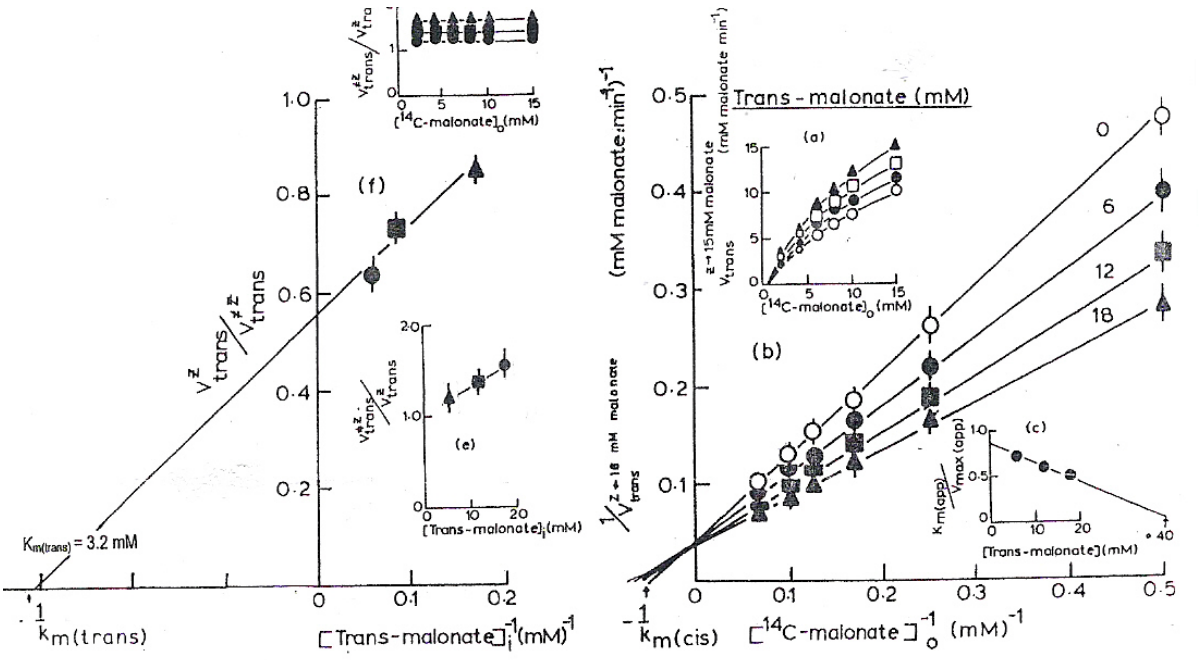


Figure 5: Effect of SITS on [^{14}C]malonate uptake
Erythrocytes (25% Hct) were incubated in [^{14}C]malonate solution and the uptake was assayed as a function of medium SITS concentrations: ●, control; ○, 0.25 mM; Δ, 0.50 mM; □, 0.10mM. Lineweaver-Burk plots for double-reciprocal of $V^{zt}_{malonate}$ vs. medium [^{14}C]malonate. Inset: Lineweaver-Burk plot slopes vs. medium SITS inhibitor concentrations.



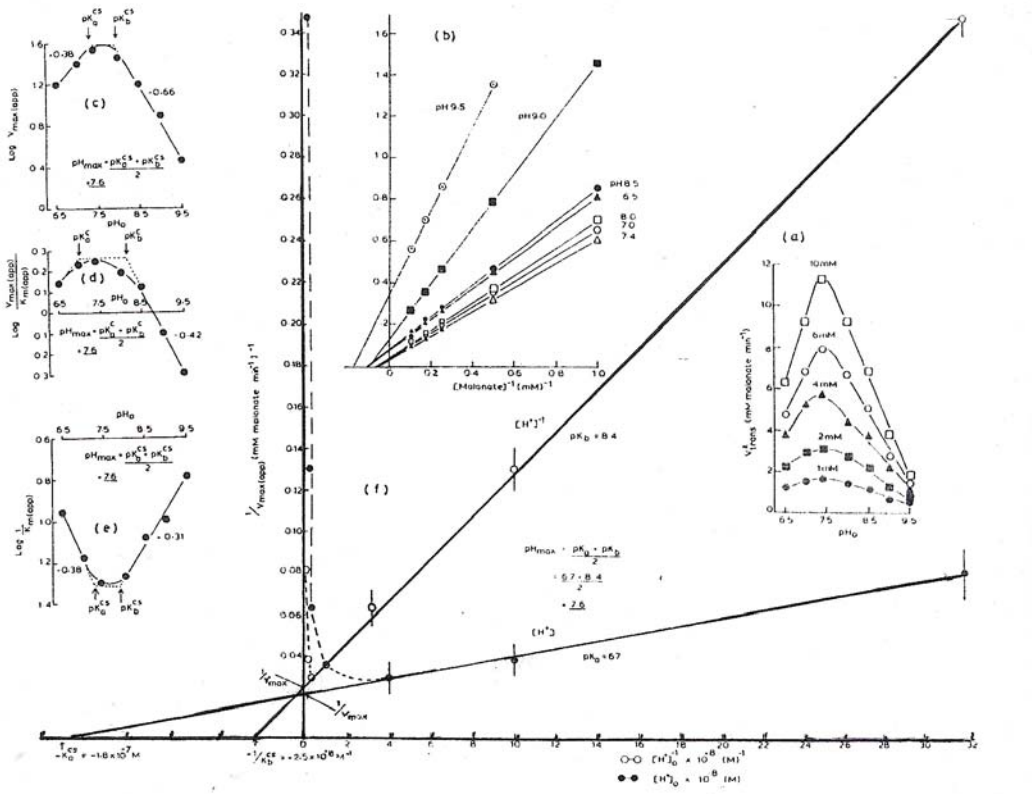
Salhany et al. (1983) reported that increasing the fixed trans-sulphate concentration caused the velocities of dithionite influx increase. Similarly, the rate of [^{14}C]malonate uptake increased with increasing trans-malonate concentration (Fig. 6).

Figure 6: Effect of [^{12}C]malonate [$_{trans}$] on the rate of [^{14}C]malonate influx.
The concentration of intracellular [^{12}C]malonate $_{[trans]}$ was ○, zero; ●, 5.9 mM; ■, 11.8 mM; ▲, 17.9 mM. The concentration of [^{14}C]malonate $_{[cis]}$ was 2 mM, 4 mM, 6 mM, 10 mM and 15 mM. (a) Initial rate of [^{14}C]malonate influx vs. Cis-[^{14}C]malonate concentrations. (b) Lineaweaver- Burk plots for double-reciprocal of initial rate of [^{14}C]malonate influx vs. medium [^{14}C]malonate as a function of trans-malonate concentrations. The value of $V^{trans}_{max(apparent)} / K^{cis}_{m(apparent)}$ was observed to increase with increasing trans-malonate concentration. © slopes of Lineweaver-Burk plots vs.trans-malonate concentration. (d) Initial rate of [^{14}C]malonate influx in the presence of trans-malonate ($V^{\neq z}_{trans}$) to that in the absence of trans-malonate (V^{z}_{trans}) vs. medium [^{14}C]malonate concentrations as a function of trans-malonate. (e) Relative increase in the initial rate of [^{14}C]malonate flux (extrapolated from d) vs. concentrations of trans-malonate. (f) Lineweaver-Burk plot for $V^{\neq z}_{trans} / V^{z}_{trans}$ vs. concentrations of trans-malonate.



The influence of medium pH on both [^{14}C]malonate influx was monitored over the pH range 6.5-9.5, a range where malonate exists predominantly as a dianion (pk_1, 2.7; pK_2, 5.3). Changes in the apparent values of both $K_{m(cis)}$ and $V_{max}^{zero\text{-}trans}$ were observed (Fig. 7b). A bell-shaped curve of log V_{max} / K_m versus pH_o resulted with maximum rate of [^{14}C]malonate uptake in the vicinity of pH_{max} 7.6 (Fig. 7d). [^{14}C]malonate efflux was also monitored over the pH range 6.5-9.5, and the membrane potential (E_m = RT/nF. $lnr_{equilibrium}$ [trans-malonate /cis-malonate]) was observed to be of least value at the vicinity of pH 7.4 (unpublished results).

Figure 7: Effect of medium pH on [^{14}C]malonate influx Erythrocytes (25% Hct.) were suspended in Glycine/HCl-NaOH buffer (1:1, v/v) and incubated at 37ºC in isoosmotic [^{14}C]malonate solutions of pH 6.5-9.5. (a) Rate of Malonate influx vs. as a function of medium malonate concentrations. (b) Lineweaver-Burk plots for double-reciprocal of rate of malonate influx vs. medium malonate concentrations. (c) Logarithm of apparent V^{zt}_{trans} vs. pH_o. (d) Logarithm of apparent V^{zt}_{trans} / apparent $K_{m\,(cis)}$ for malonate influx vs. pH_o. (e) Minus logarithm of apparent $K_{m\,(cis)}$ vs. pH_o. (f) Reciprocal of apparent V^{zt}_{trans} vs. both $[H^+]$, ●-● and reciprocal $[H^+]$, ○-○. The dashed lines correspond to the situation where the inequalities of $[H^+] << K_A^{carrier}$, $[H^+] << K_A^{carrier\text{-}malonate}$ no longer hold.



Discussion

The membrane band 3 protein,AE1, is multifunctional, and plays several varied and important functional and structural roles in the red blood cell, but its principal biological role is [Cl-] – [HCO_3-] exchange.

The structure of dicarboxylate anions governs their ability to penetrate erythrocyte membranes, although both permeant and impermeant species inhibit [^{14}C]malonate for the AE1 band 3 protein (Hajjawi and Hider, 1987). The anion transport process in human erythrocytes shows a clear specificity with an intercharge distance of 4 Å, and it is qualitatively similar to that reported for bovine erythrocytes (Giebel and Passow, 1960; Aubert and Mutais, 1975; Jennings and Adam, 1996).

One of the most striking aspects of the results presented above is the self inhibitory potency of malonate and it is so dependent on the side of the membrane to which it is added. The $K_{m(cis)}$ for [^{14}C]malonate influx was shown to be 21.6 mM (Fig.2), whereas $K_{m(trans)}$ [^{14}C]malonate efflux was shown to be 3.2 mM (Fig. 6). One interpretation of the Michaelis constant,K_m, is that it equals the concentration of substrate at which 50% of the "anion transporter" active sites are occupied by substrate. Interestingly enough, hexokinase K_m is 0.15mM for glucose, and 1.5 mM for fructose. Hence, the lower the K_m, the higher the affinity. Such higher affinity is distinguishable between the $outer_{cis}$ and $inner_{trans}$ anion transporter (Fig.2 and Fig. 6.f). Schnell et al. (1978), Salhany (1998), Knauf

et al. (2002) and Chernova et al (2008) have reported similar asymmetry for Cl^- and Cl^-/HCO_3^- heteroeexchange rates. Fig.3 shows a $K_{i(cis)}$ of 21 mM on [^{14}C]malonate efflux which is the $K_{m(cis)}$, i.e. a trans membrane direct mediated anion transporter for both anion influx and anion efflux. Salhany et al. (2006) shows that there is an interaction between a primary stilbene disulfonate binding site and a stilbenesulfonate efflux site.

Both BPS (Fig. 4) and SITS (Fig. 5) show that they are competitive inhibitors to [^{14}C]malonate influx with K_i of 0.7mM and 1mM, respectively. Arginine specific reagents are found to be powerful inhibitors of dianion exchange in the red blood cell membrane. The reversibility of BPS (Fig. 4.d_3) is consistent with the reported findings of Janas et al. (1989), Cabanchick and Greger (1992), Salhany (1995), and Guizouarn et al. (2001). The effect of medium pH on [^{14}C]malonate influx shows a pK_a of 6.7 and pK_b of 8.4 (Fig.7.f) which is indicative of α-amino acid(s). A near physiological pK_a value of 6.8 had been also reported by Denicola et al. (1998). The complexity of interpreting these bell-shaped curves (Fig.7.a) might be attributed to a reduction in the cytoplasmic ionic strength changes the interaction of AE1 and other cytoplasmic proteins, forming tetrameric channel (Perlman and Goldstein, 2004). Also, the Lineweaver-Burk plots for [^{14}C]malonate influx as a function of medium pH (Fig.7.b) shows characteristics of a carrier rather than a channel for AE1 with an allosterically linked two interacting sites and an essential ternary complex (Salhany and Rauenbuehler,1983; Jannings,1995; Salhany,1995). The experimental values of both pK_a and pK_b (Fig. 7 c, d, and e) may account to several arginine residues located in a tight cleft that makes permeation of *trans* dicarboxylates very awkward, yet it is accessible competitively to both SITS and BPS.

Until we have good structural data, more of good kinetic data, and more electrophysiology on the expressed AE1, we are unable to come to any molecular detail or mechanism to explain the transport mode in an abundant membrane protein. Nevertheless, the recognition of dual properties as a carrier and as a channel of modified band 3 protein seems to be secure (Ellory et al., 2009). It is well established that AE1 is a stable dimmer (Casey and Reithmeier, 1991; Wang et al., 1994; Knauf et al., 2002), and there are considerable data in favour of allosteric interactions in the functioning of AE1 (Salhany, 1996; Salhany, 2003). Hence, the possible role of subunit interactions in anion transport has been the subject of debate (Jennings, 2005). We have however ongoing studies on [14C]malonate dianion flux kinetics in human erythrocyte cells. The preliminary results are promising to provide more evidence in support of a carrier and the likelihood of a sequential anion exchange mechanism (Verkman et al., 1983; Salhany, 1998; Chernova et al., 2008), rather than a ping-pong model (Passow, 1986; Knauf et al., 2000).

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