Review Letter

# IDENTIFYING THE MONOSACCHARIDE TRANSPORT PROTEIN IN THE HUMAN ERYTHROCYTE MEMBRANE

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## 1. Introduction

Monosaccharides permeate the human erythrocyte membrane by a mechanism of facilitated diffusion which has been extensively investigated [1–3]. Many of the earlier studies were concerned with kinetics [4] but there is an increasing interest in identifying the membrane proteins involved in the transport process by means of reconstitution experiments in liposomes [5–9] and planar lipid bilayers [10–14]. Fig.1 shows a polyacrylamide gel polypeptide profile of human erythrocyte membrane proteins. Many very similar profiles have been reported in the literature (see below). In terms of the notation of Fairbanks et al. [15] for the designation of the polypeptide bands, band 3 and the region between bands 4.2 and 5, the so-called 4.5 region, have been proposed as components of the monosaccharide transport system. The association of band 3 with glucose transport was implicated by binding experiments [16–18] and reconstitution studies [6,11–14] whereas other investigations have led to the involvement of region 4.5 [5,7,8,19,20]. Band 3 is a heavily stained region of the electrophoretogram which contains the transmembrane polypeptides associated with anion permeability [21–24], ATPase activity [25,26] and cytochalasin B binding [17]. The structural features and disposition of the band 3 polypeptides have been investigated in some detail [27–29]. In contrast the 4.5 region is relatively lightly stained. Using the procedure in [15] for SDS–PAGE (sodium dodecylsulphate–polyacrylamide gel electrophoresis) this region usually consists of a number of overlapping bands whereas the Laemmli procedure [30] gives better resolution to reveal 6–7 bands [31,32] as seen in fig.1. While the bulk of the experimental evidence implicates band 3 or region 4.5, 'spectrin' (bands 1,2) has also been related to glucose transport [33].

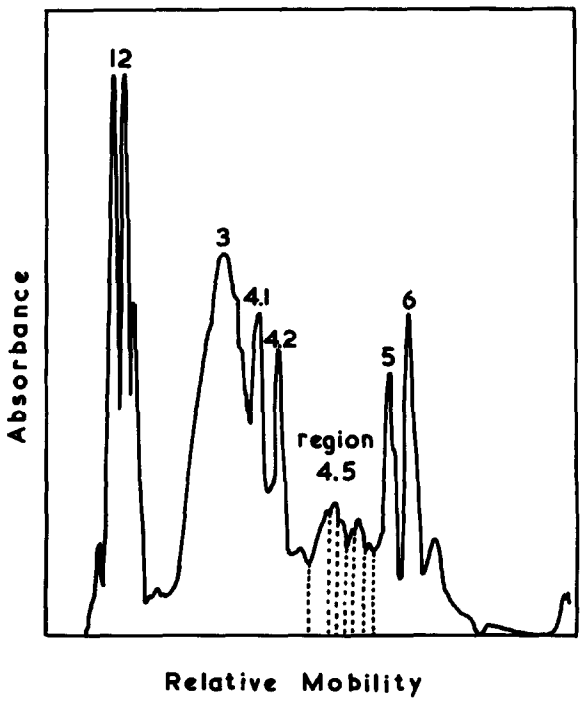


Fig.1. Electrophoretogram of human erythrocyte membrane proteins as obtained by the Laemmli procedure [30]. The notation of the bands is that of Fairbanks et al. [15].

A necessary requirement in the identification of the polypeptides involved in monosaccharide transport is that the number of such polypeptides must be sufficiently large to account for the number of transport sites in the membrane. It is thus important to accurately establish both the number of transport sites and the amounts of particular polypeptides believed to be involved in transport. While these points have been

considered in relation to band 3 polypeptides there has been no detailed attempt to analyse region 4.5.

Here the current situation is reviewed with respect to these two factors with particular reference to the polypeptides which constitute band 3 and region 4.5.

## 2. Number of transport sites and membrane composition

### 2.1. *Estimation of the number of transport sites*

#### 2.1.1. Estimates based on kinetic parameters

Several kinetic models for monosaccharide transport in erythrocytes have been proposed to explain the differences in the values of the kinetic parameters ($K_m$ and $V_{max}$) as determined under various experimental conditions [3]. We shall consider only those experimental conditions corresponding to unidirectional flux of monosaccharide. These are the zero-*trans* exit (*zt*ex) and entry (*zt*en) experiments in which the rate of efflux or influx of a sugar through the erythrocyte membrane is measured and to a first approximation can be represented by Michaelis-Menten kinetics. It is more difficult to interpret equilibrium exchange experiments in which the sugar is transported in both directions and which requires more complex kinetic models involving high and low affinity binding sites and consequently more kinetic parameters. Estimates of the number of transport sites per cell can be made as follows.

The flux $J$ (mol . cm$^{-2}$ . s$^{-1}$) of a monosaccharide across the cell membrane is related to the net transport rate $v$ (mol . (cm$^3$ cell water)$^{-1}$ . s$^{-1}$) and membrane permeability, $P$ (cm . s$^{-1}$), by the equation:

$$J = v\left(\frac{V_c}{A_c}\right) = P(S_o - S_i) \qquad (1)$$

where $V_c$ and $A_c$ are the volume of water per cell and membrane area, respectively, and $S_o$ and $S_i$ are the monosaccharide concentrations (mol . cm$^{-3}$) on the outside and inside of the cell. The net transport rate into the cell is given by:

$$v_{o\to i} = \frac{V_{max}\, S_o}{K_m + S_o} - \frac{V_{max}\, S_i}{K_m + S_i} \qquad (2)$$

It follows from eq. (1) and (2) that the permeability is given by:

$$P = V_{max}\, K_m \left(\frac{V_c}{A_c}\right) \frac{1}{(K_m + S_o)(K_m + S_i)} \qquad (3)$$

Eq. (3) shows that if the system is represented by Michaelis-Menten kinetics the permeability as expressed in eq. (1) is dependent on the monosaccharide concentrations on either side of the membrane, however, the maximum permeability will be given in the limit when either $S_o = 0$ and $S_i \to 0$ (zero-*trans* exit) or $S_i = 0$ and $S_o \to 0$ (zero-*trans* entry). For these limits:

$$P_{max} = \frac{V_{max}}{K_m}\left(\frac{V_c}{A_c}\right) \qquad (4)$$

The maximum permeabilities calculated from eq. (4) can be used to estimate turnover numbers $t_n(s)$ from the relation $t_n = d/P_{max}$ where $d$ is the pathlength of the sugar through the membrane.

It should be noted that under conditions of equilibrium exchange the permeability is larger than for unidirectional flux. This can be interpreted as a greater rate of translocation of the 'loaded carrier' or alternatively in terms of the Singer model of transport [85], which involves a permeant-induced conformational change in a dimeric transmembrane protein, as a greater frequency of conformational change due to the presence of finite sugar concentrations on both sides of the membrane. Under these conditions the transport protein would be expected to display a larger turnover number. This does not necessarily imply a larger number of high affinity transport sites per cell (although under these conditions postulated lower affinity sites could be involved). For this reason it is stressed that the concept of turnover numbers as used here is only applicable under the limiting conditions of unidirectional flow.

The number of transport sites per cell is given $V'_{max}$ (molecules . cell$^{-1}$ . s$^{-1}$) . $t_n$. In converting literature values of $V_{max}$ from μmol . (cm$^3$ cell water)$^{-1}$ . min$^{-1}$ to $V'_{max}$ (molecules . cell$^{-1}$ . s$^{-1}$) we have taken the volume of water in the red cell ($V_c$) to be $66 \times 10^{-12}$ cm$^3$. The latter figure is based on a total cell volume of $90 \times 10^{-12}$ cm$^3$, a haemoglobin content of 35% (w/v) and a partial specific volume of 0.75 cm$^3$ . g$^{-1}$ for haemoglobin [34]. The area of the red cell membrane was taken as $155 \times 10^{-8}$ cm$^2$ [34] and the pathlength as 7.5 nm (i.e., the bilayer thickness) [47]. Estimates of the number of sites .

Table 1
Estimation of the number of monosaccharide transport sites in the human erythrocyte membrane by kinetic methods

| $K_m$ (mM) [ref.] | $V'_m$ (molecules . cells$^{-1}$ . s$^{-1}$) X $10^{-8}$ | Sugar/method | $P_{max}$ . $10^5$ (cm . s$^{-1}$) | Sites . cell$^{-1}$ . $10^{-6}$ |
|---|---|---|---|---|
| 25 [3] | 0.855 | Glucose/ztex | 0.366 | 18 |
| 240 [3] | 1.69 | Galactose/ztex | 0.075 | 167 |
| 1.6 [3] | 0.239 | Glucose/zten | 1.60 | 1.1 |
| 31.76 [3] | 1.90 | Galactose/zten | 0.064 | 22 |
| 12.7 [70] | 0.265 | Galactose/zfen | 0.224 | 8.9 |
| 74.4 [70] | 1.60 | Galactose/ztex | 0.230 | 52.1 |

cell$^{-1}$ are given in table 1 from measurements of glucose and galactose transport. There are considerable variations in $K_m$ and $V_{max}$ found by different workers [3]. The zero-*trans* exit method gives on average, ~80 X $10^6$ sites . cell$^{-1}$ and the zero-*trans* entry gives ~10 X $10^6$ sites . cell$^{-1}$. The maximum permeabilities (~$10^{-5}$ cm . s$^{-1}$) are three orders of magnitude larger than the permeabilities of bilayer lipid membranes to D-glucose [10,35,36] in the absence of mediating membrane protein.

2.1.2. Estimates based on specific binding experiments

Table 2 records a collection of estimates of the number of binding sites . cell$^{-1}$ which have been measured by direct methods such as equilibrium dialysis and differential labelling for D-glucose and substances which can be made to interact relatively specifically with sugar transport sites in the erythrocyte membrane. Cytochalasin B competitively inhibits sugar transport and has been shown to have stereochemical features which are almost identical to the C1-conformation of β-D-glucopyranose [52]. The binding of cytochalasin B is not however as simple as might be thought. Jung and Rampal [48] found three major groups of binding sites with different dissociation constants and populations. Site I the highest affinity site, of which there are 1.8 X $10^5$ . cell$^{-1}$ was originally associated with the glucose transport 'carrier' but later work [49] showed that site II (0.54 X $10^5$ sites . cell$^{-1}$) when liganded interacts with site III (0.36 X $10^5$ sites . cell$^{-1}$) which then becomes glucose-sensitive.

Table 2
Estimation of the number of monosaccharide binding sites in the human erythrocyte membrane by direct methods

| Authors [ref.] | Method | Identification of binding site | Sites . cell$^{-1}$ |
|---|---|---|---|
| Levine and Stein (1967) [37] | ED (D-glucose) | – | 1.5 X $10^6$ |
| Kahlenberg et al. (1971) [38] | Preferential D-glucose binding | – | 1.9–2.5 X $10^5$ |
| Masiak and Le Fevre (1972) [39] | ED (D-glucose) | – | <$10^5$ |
| Taverna and Langdon (1973) [40] | AL (D-glucosyl isothiocyanate) | Bands 3,4 | 3.0 X $10^5$ |
| Taverna and Langdon (1973) [41] | Cytochalasin B binding | – | 3.3 X $10^5$ |
| Eady and Widdas (1973) [42] | DL (FDNB) | – | ⩾$10^6$ |
| Lin and Spudich (1974) [43,44] | Cytochalasin B binding | Band 3 | 3.0 X $10^5$ |
| Jung and Carlson (1975) [45] | DL (FDNB) | ~180 000 mol. wt protein | 3.0 X $10^5$ |
| Basketter and Widdas (1976) [46] | Cytochalasin B binding | – | 2.4 ± 0.3 X $10^5$ |
| Batt et al. (1976) [19] | DL (glutathione-maleimide) | Band 4.5 | 2.8–4.8 X $10^4$ |
| Jung et al. (1977) [48,49] | Cytochalasin B binding | Bands 3,4.2, region 4.5 | 1.8 X $10^5$ (site I) |
| Lienhard et al. (1977) [50] | DL (FDNB) | Region 4.5 | 3.5 X $10^5$ |
| Shanahan and Jacquez (1978) [51] | DL (FDNB) | Bands 1,2 | 2900 |
| | | Band 3 | 8700 |
| | | Region 4.5 | 3.36 X $10^4$ |

*Abbreviations:* ED, equilibrium dialysis; AL, affinity labelling; DL, differential labelling

The differential labelling experiments involve incubation of the labelled reagent (e.g., [$^{14}$C]- or [$^{3}$H]FDNB) with two identical aliquots of cells in the presence of a reagent which has a potentiating effect on the reaction with the label (e.g., [$^{14}$C]FDNB with D-glucose [45] or 2-deoxyglucose [42]) and in the presence of a reagent which has a protective effect on the reaction with the label (e.g., [$^{3}$H]FDNB with ethylidene glucose [42], cytochalasin B or D-maltose [51]). Krupka has reported a detailed study of the effects of a range of reagents on the rates of inhibition of sugar transport by FDNB [53] and classified them according to whether they expose or protect the transport sites to reaction with FDNB.

The first direct observation of selective binding of D-glucose by ghost protein extracts by equilibrium dialysis was reported by Levine and Stein [37] who obtained $1.5 \times 10^6$ sites . cell$^{-1}$ but Masiak and Le Fevre failed to repeat this work using an identical method. Apart from this first estimate and the more recent differential labelling experiments of Batt et al. [19] and Shanahan and Jacquez [51] the data in table 2 show reasonable concordance and lead to $\sim 10^5$ sites . cell$^{-1}$.

It is significant that the two lower estimates of $\sim 10^4$ sites . cell$^{-1}$ are specifically associated with labelling of protein in region 4.5 of the ghost electrophoretogram pattern. In [54] Baldwin et al. purified a cytochalasin B binding component with mol. wt 55 000 (to be found in region 4.5). From the experimental details in [54] we estimate also $\sim 10^4$ binding sites . cell$^{-1}$. In this connection it is noteworthy that Lin and Snyder [55] found $5 \times 10^4$ high affinity sites for cytochalasin B on the cytoplasmic side of the membrane which were unrelated to sugar transport.

Comparison of the number of sites . cell$^{-1}$ in tables 1 and 2 clearly shows that the kinetic methods lead to values at least an order of magnitude larger than the direct binding methods. The permeabilities given in table 1 are in accord with previous estimates from kinetic methods [56,57]. The numbers of sites would be in line with the values given by the binding studies if we were to assume that the sugar molecules have a pathway through the membrane which was of the order of 1/10th the thickness of the membrane. Such an assumption would not be inconsistent with the molecular models envisaged for mediated transport [1,71].

### 2.2. *Polypeptide composition of the erythrocyte membrane*

Steck [59] has reported estimates of the percentage of the major erythrocyte polypeptides based on Coomassie blue-stained gels. These figures were the result of an analysis of 21 gel patterns but they did not include the 4.5 region of the electrophoretogram. In order to make an assessment of the percentage of polypeptide in region 4.5 we chose to analyse a number of densitometric scans of SDS–polyacrylamide gels reported in the literature. Our procedure involved photographing and enlarging the reported scans followed by graphical integration based on weighing. The results are shown in table 3 and lead to average values for band 3 and region 4.5 of ~24% and ~10%, respectively. The average value for band 3 is in agreement with the 24% quoted in [59] so that we can have considerable confidence in this value. It is noteworthy that the percentage of polypeptide in band 3 remains constant during ageing of the red cell membrane both in vivo and in vitro [68].

The results in table 3 are based largely on SDS–PAGE procedures [15] which in general lead to a 4.5 region consisting of between 2 and 4 poorly resolved bands, however, the Laemmli procedure [30] gives gel patterns showing at least 6 faint but distinct bands [31,32]. We have taken the gel profiles reported by Morrison et al. [31,32] photographed and scanned them and together with our own gels obtained by the Laemmli method we have attempted to estimate the percentage of polypeptide in the six major bands in region 4.5. The results calculated from the total average value of 9.8% (table 3) and the relative areas of the bands in region 4.5 are given in table 4, together with molecular weight estimates and the number of polypeptides . cell$^{-1}$ assuming a protein content of $6 \times 10^{-13}$ g . cell membrane$^{-1}$ [69]. The errors in the latter figures are based on the discrepancies in the percentages of each band.

## 3. Discussion and conclusions

The data in table 3 give $9.6 \pm 1.4 \times 10^5$ molecules of band 3 and $6.9 \pm 1.4 \times 10^5$ molecules of band 4.5 per cell. These estimates are based on mol. wt 90 000 and 51 000, respectively and $6 \times 10^{-13}$ g membrane protein . cell$^{-1}$. Excluding the very low figures given in [19,51] the binding studies in table 2 lead to an estimate of $\sim 3 \times 10^5$ monosaccharide binding sites cell$^{-1}$. There are clearly enough molecules of either

Table 3
Polypeptide composition of human erythrocyte membranes

| Authors [ref.] | % Band 3 | % Region 4.5 |
|---|---|---|
| Fairbanks et al. (1971) [15] | 23 | 9 |
| Steck and Dawson (1974) [58] | 27 | 13 |
| Lin and Spudich (1974) [44] | 26 | 8 |
| Steck (1974) [59] | 23 | 9 |
| Lin et al. (1975) [60] | 25 | 12 |
| Brown et al. (1975) [61] | 18 | 8 |
| Kahlenberg and Walker (1976) [16] | 20 | 11 |
| Zala and Kahlenberg (1976) [6] | 22 | 10 |
| Pickard and Paterson (1976) [62] | 23 | 5 |
| Lienhard et al. (1977) [50] | 21 | 10 |
| Zoccoli and Lienhard (1977) [63] | 21 | 10 |
| Kadlubowski (1978) [68][a] | 27 | 9 |
| Kadlubowski (1978) [68][b] | 31 | 13 |
| Kadlubowski (1978) [68][c] | 26 | 11 |
| Kadlubowski (1978) [68][d] | 28 | 9 |
| Luthra et al. (1978) [65] | 27 | 9 |
| Jones and Nickson (1978) [13] | 19 | 11 |
| Galleti et al. (1978) [66][e] | 14 | 14 |
| Vimr and Carter (1976) [67][f] | 19 | 14 |
| Average and standard deviation (excluding [66] and [67]) | 23.9 ± 3.5 | 9.8 ± 1.9 |

[a]Young cells (5% gel); [b]old cells (5% gel); [c]young cells (7.5% gel); [d]old cells (7.5% gel); [e]rat erythrocytes; [f]rabbit erythrocytes

band 3 or band 4.5 (in total) to account for this number of binding sites. If on the other hand we accept the heterogeneity of band 4.5 then the results in table 4 would indicate that there is not quite enough of any one sub-band of region 4.5 to account for $3 \times 10^5$ binding sites. The low values $\sim 10^4$ sites . cell$^{-1}$ found in some of the differential labelling studies [19,51] could be accounted for by any of the sub-bands of region 4.5. It should however be noted that in differential labelling experiments we are dealing with non-equilibrium systems in which the difference in the rates of reaction of the label with the binding site in the presence of potentiating and protecting substances is maximised and even when this has been achieved the experiment has a predilection towards a minimum estimate.

Several membrane functions have been attributed to band 3 protein, these include water transport [61], anion transport [21–24], cation transport [73], ($Na^+$, $K^+$)-ATPase activity (150 molecules . cell$^{-1}$) [25,26,

Table 4
Polypeptide composition of region 4.5 of the human erythrocyte membrane electrophoretogram

| Authors [ref.] | Band designation | | | | | |
|---|---|---|---|---|---|---|
| | $4.5_1$ | $4.5_2$ | $4.5_3$ | $4.5_4$ | $4.5_5$ | $4.5_6$ |
| King and Morrison (1977) [31] | 1.8 | 1.7 | 1.1 | 1.7 | 1.7 | 1.8 |
| Mueller and Morrison (1977) [32] | 1.4 | 2.0 | 0.87 | 1.8 | 1.5 | 2.3 |
| This work | 2.9 | 2.5 | 1.1 | 0.80 | 1.8 | 0.70 |
| Mean | 2.0 | 2.1 | 1.0 | 1.4 | 1.7 | 1.6 |
| Mol. wt $\times 10^{-3}$ | 55 | 53 | 52 | 51 | 48 | 45 |
| No. molecules . cell$^{-1}$ $\times 10^{-5}$ | 1.3 ± 0.6 | 1.4 ± 0.3 | 0.69 ± 0.9 | 0.99 ± 0.4 | 1.3 ± 0.2 | 1.3 ± 0.7 |

74] cholinesterase activity (6000 molecules . cell$^{-1}$) [15], concanavalin A binding [72,75], and diisothiocyanostilbene disulphonate (DIDS) binding (1–1.2 × $10^6$ sites . cell$^{-1}$) [76].

In terms of numbers of molecules . cell$^{-1}$ the major proportion of band 3 appears to be involved in anion transport. The heterogeneity of band 3 is believed to arise primarily from variability in carbohydrate content [28] rather than from heterogeneity in the polypeptides. It is conceivable that band 3 protein contains binding sites for both anions and monosaccharides. In relation to this possibility it is known that both anion and monosaccharide transport are inhibited by phloretin and phlorizin [77,78]. Anion transport can be inhibited by proteolytic attack by non-specific enzymes such as pronase and papain [76] but it is not inhibited by trypsin or chymotrypsin [79]. However, monosaccharide transport appears considerably less sensitive to inhibition by trypsin [80] or pronase [81]. These observations could indicate that the anion and monosaccharide binding sites are on separate proteins or that they are in different regions of the same protein. Crucial to the role of band 3/4.5 in monosaccharide transport is the disposition of the membrane proteins, here the transmembrane disposition of band 3 is not in question [27–29,82] whereas there is less evidence to support the transmembrane disposition of band 4.5 protein [19].

There is increasing evidence that band 3 polypeptides can be degraded by membrane-associated proteases both in ghosts [83] and in Triton X-100 extracts of ghosts [14,84] to give a transmembrane fragment with mol. wt ~55 000 which appears in region 4.5 of the gel pattern and is consistent with cleavage of band 3 at its cytoplasmic interface. This cleavage may not impair transport activity of the protein and as suggested [14] could reconcile the conflicting evidence on the identification of the transport protein. Alternatively monosaccharide transport may require more than a single membrane protein, and perhaps involves both a portal (integral) protein and a peripheral component [85]. The ease with which band 3 can be crosslinked in the membrane to give a dimer [86] make it a likely candidate for a portal protein of the type envisaged by Singer [85].

If monosaccharide transport involves a component of region 4.5 then it is imperative that more information on the disposition of the proteins in the membrane is forthcoming, that the interaction (if any) between anion and sugar transport is investigated further and that future experiments are carried out with cognisance of the possible artifactual origin of polypeptides in region 4.5 arising from proteolysis.

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**Note added in proof**

The recent work of R. E. Mullins and R. G. Langdon [Biochemistry (1980) 19, 1119–1205; 1205–1212] strongly supports the argument that the glucose transporter of mol. wt 55 000 [7,8,54] arises from the degradation of a band 3 polypeptide.

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