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Integral band 7 protein of the human erythrocyte membrane
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Introduction
In 1961, Lock, Sephton-Smith and Hardisty [1] coined the term 'stomatocyte' to describe the red cell morphology in a novel congenital haemolytic anaemia. Rather than presenting a discoid shape, the cells showed a linear slit of central pallor, around which the cell folded in like a mouth, or stoma. This trait was inherited as an autosomal dominant. The early haematological tests suggested an abnormality in cell volume control by the membrane, and cation transport studies [2] showed that these cells had a major defect in membrane permeability to the univalent cations Na+ and K+. Other cases and variants showing less marked ion transport abnormalities, and less marked haemolysis, were described (see [3, 4]). The clinical condition is unusual in that abnormal membrane transport can easily be shown to play a major role in the pathophysiology. Unlike cystic fibrosis, a much more common disease also involving membrane transport [5], the functional cell is easily available for biopsy and meaningful physiological and biochemical study; an unusual event in clinical medicine.

In 1981 Lande and others [6] found that a previously uncharacterized membrane protein, which showed a mobility in the 'Band 7' region [7] at around 30 kDa on SDS gels, was deficient in the membranes of these cells. This was illustrated in Fig. 1. Thus a disorder of ion transport was now associated with a clear protein abnormality. While the majority of transport-related proteins so far purified and cloned are the actual transporters, such as the Na+K+ pump and the Na+ and K+ channels of nerve [8], this protein, which is absent in leaky cells, seems unlikely to act as a transporter itself; rather, it would appear to act as a 'plug', blocking a channel which is tight in normal cells. Few such proteins had previously been described.

Cation transport across the human red cell membrane
The regulation of univalent cation transport across the human red cell membrane serves to control intracellular osmolarity. A net intracellular deficit is maintained by the balance between the ATP-driven, ouabain-inhibitable, Na+K+ pump and a 'passive leak': a non-selective, bidirectional, non-saturable process resembling passive diffusion which is sensitive to only the least selective of inhibitors such as external Mg2+ [9]. Tracer flux studies reveal a number of other pathways such as Na+K+Cl- co-transport, Na+Na+ exchange, a Ca2+-activated K+ transport and K+Cl- co-transport (see [10]), but in normal human red cells under physiological conditions, these pathways are either silent or mediate only exchange fluxes, although similar systems do have extremely active roles in other cell types [8].

The molecular pathway for this background leak is unknown. While superficially uninteresting, its proper control (and the precise Na+:K+ selectivity) is an essential prerequisite for normal cell volume regulation in all human cells and for a long list of cell functions, such as action potential propagation, sensory transduction, and control of Ca2+ permeability via voltage-dependent Ca2+ channels. While an increased leak can be compensated for by increased Na+K+ pump activity (to a point), such activity is a futile waste of energy. Other systems, notably the 'short term' volume regulators (Na+K+Cl- co-transport, K+Cl-, Na+H+ exchange) can no doubt compensate but also represent a wasteful dissipation of energy.

Abbreviations used: HSt, hereditary stomatocytosis; DMA, dimethyl adipiminate; SITS 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; DIOA, [(dihydroindenyl)oxy]alkanoic acid.
Two-dimensional protein analysis of red cell membranes

Membranes were prepared by hypotonic lysis [34] and solubilized in 9 M-urea and 1% NP40. Non-equilibrium pH gradient isoelectric focusing was performed according to OFarrell [35]. In the first dimension, an 8 cm gel was electrophoresed for 4 h at 400 V. After equilibration in SDS and mercaptoethanol, the proteins were run on a 12% polyacrylamide gel, which was then silver stained. Top, stomatocytic cells; bottom, normal control. The location of the missing protein is arrowed. Abbreviation used: TD, tracking dye.

These points are relevant to HSt. The steady-state intracellular cation concentrations, when measured by flame photometry, show a very marked abnormality such that intracellular [Na+], normally about 10 mmol/l of cells, is found to be approximately 80; while [K+], normally 100, is reduced to 30 [11]. Tracer K+ influx is a useful 'assay' for ion transport in red cells: that fraction which is inhibited by ouabain is mediated by the Na⁺K⁺ pump; 0.1 mM-bumetanide inhibits the Na⁺K⁺Cl⁻ co-transport system. In the presence of both of these inhibitors, the residual flux reflects the 'linear leak', although there can be a small ouabain + bumetanide-sensitive K⁺Cl⁻ co-transport component which is different from Na⁺K⁺Cl⁻ co-transport. In these patients, the Na⁺K⁺ pump flux is about 20-times normal. Na⁺K⁺Cl⁻ co-transport is low-normal (this system contributes only exchange fluxes) while the residual flux is greatly increased, like the Na⁺K⁺ pump. These data can be interpreted in terms of a 'primary' leak only partly compensated for by an increased Na⁺K⁺ pump. Ouabain binding studies reveal an increased number of Na⁺K⁺ pump sites [12], which could represent increased Na⁺K⁺ pump synthesis at the cell synthesis stage. It should also be noted that Na⁺K⁺Cl⁻ co-transport is low, consistent with cellular overhydration.

The stomatocytic leak flux is not selective for Na⁺ over K⁺ and shows no rectification. Ca²⁺ fluxes are not increased. This leak flux shows near-linear dependence on external [K⁺]. If saturation is occurring, the Kₘ is in excess of 50 mm, typical of channels in excitable tissue [13]. (The Na⁺ dependence is difficult to interpret because of the presence of the saturable Na⁺Na⁺ exchange component.) The temperature dependence shows monotonic dependence, unlike normal cells which show a minimum at about 8°C [14], showing a qualitative as well as quantitative difference between HSt and normal cells. It is not simply a matter of 'more holes'; they are physically different. It could be that the flux is carried by a known molecule working in an abnormal mode, unregulated. Tests of different inhibitors, previously characterized in red cells and other systems, reveal no significant effects (see Table 1). Of course, non-binding of an inhibitor does not rule out the participation of that system or a subset of it, since the inhibitor may not bind to an abnormally modified molecule.

While the standard ion channel inhibitors have no effect, one substance that does inhibit is a protein cross-linking reagent, dimethyl adipiminate (DMA) [15]. The effect is shown in Fig. 2. In the absence of DMA treatment, both Na⁺K⁺ pump (ouabain-sensitive) and residual (ouabain plus bumetanide-resistant) K⁺ influxes are increased, consistent with the known pathophysiology of the condition [2,12]. At 1 mM, DMA markedly inhibits the residual (leak) K⁺ influx in the abnormal cells but has no inhibitory effect on the equivalent flux in the normal cells, and in fact marginally increases the flux at 10 mM. The DMA has relatively little effect...
Table 1
Previously characterized inhibitors of Na⁺ or K⁺ transporters or channels with no significant inhibitory effect on the ouabain plus bumetanide-resistant K⁺ influx in stomatocytic red cells

Concentrations all 0.1 mM except where stated. If no reference given, see [13].

<table>
<thead>
<tr>
<th>Substance</th>
<th>Target system</th>
</tr>
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<tbody>
<tr>
<td>Ouabain</td>
<td>Na⁺K⁺ pump [10]</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>Na⁺K⁺Cl⁻ co-transporter [27]</td>
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<tr>
<td>DIOA</td>
<td>K⁺Cl⁻ transport inhibitor [29]</td>
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<tr>
<td>Amiloride</td>
<td>Na⁺ channel inhibitor [29]</td>
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<tr>
<td>Methyl amiloride</td>
<td>Na⁺-H⁺ exchanger [29]</td>
</tr>
<tr>
<td>SITS</td>
<td>Band 3 anion exchanger [10]</td>
</tr>
<tr>
<td>DIDS</td>
<td></td>
</tr>
<tr>
<td>Phloretin</td>
<td>Glucose transporter [30]</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Gardos (Ca²⁺-activated K⁺) channel [31]; ‘A’ channel excitatory tissue</td>
</tr>
<tr>
<td>Apamin</td>
<td>Ca²⁺-activated K⁺ channels in nerve</td>
</tr>
<tr>
<td>Charybdotoxin</td>
<td>Ca²⁺-activated K⁺ channels in normal red cells</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>Voltage-gated Na⁺/K⁺ channel in normal red cells</td>
</tr>
<tr>
<td>Ba⁺⁺ (1 mM)</td>
<td>K⁺ channels, non-specific</td>
</tr>
<tr>
<td>Procaine</td>
<td>Na⁺ channel; endplate channel; class I antiarrhythmic</td>
</tr>
<tr>
<td>Gallamine</td>
<td>Muscarinic antagonist</td>
</tr>
<tr>
<td>Strychnine</td>
<td>Na⁺ channels</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>Most K⁺ channels</td>
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<tr>
<td>(1 mM)</td>
<td></td>
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<tr>
<td>Glibenclamide</td>
<td>ATP-stimulated K⁺ channel in pancreatic islet cells</td>
</tr>
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<td></td>
<td>[33]</td>
</tr>
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on the ouabain-sensitive Na⁺K⁺ pump or the bumetanide-sensitive Na⁺K⁺Cl⁻ co-transport influxes in either normal or abnormal cells. Other cross-linkers, including BS3 and DST, had no effect. It could be that DMA sits astride the putative channel, blocking it, or it could be that the cross-linker stabilizes an oligomeric structure.

Membrane structure
The human red cell membrane is particularly well-studied in terms of protein composition and structure. Two-dimensional analyses show approximately 200 different proteins, of which about 10 are present in substantial quantities (>100,000 copies per cell). A most useful operational and functional classification of the proteins can be made into 'integral' and 'peripheral' proteins, based on solubility in detergents or in media of low or high ionic strength. Those which are not solubilized by detergent but soluble after ionic strength manipulation (spectrin, ankyrin, actin, Band 4.1) form the cytoskeleton, a network-like protein structure lying on the cytoplasmic side of the lipid bilayer. Those which are detergent-soluble but not susceptible to changes in ionic strength (Band 3, glycophorin) are strongly associated with the lipid bilayer itself and are hydrophobically anchored to it by one or more membrane-spanning domains.

The different bands on the standard Laemmli SDS gel of normal human red cells were numbered by Fairbanks [7], and the principal components are now well characterized [16]. Spectrin makes up bands 1 and 2; band 3 is the anion exchanger; band 4 comprises many proteins including the main sub-
Fig. 2
Effect of DMA on K⁺ influx in normal and stomatocytic cells

The cells were washed in media containing (mM): Na⁺, 150; Cl⁻, 150; Mops, 15 (pH 7.4 at 20°C); glucose 5. The cells were treated with DMA (1 and 10 mM) for 10 min at room temperature then washed twice in the above medium. The cells were dispensed to triplicate Eppendorf tubes containing, if required, ouabain or bumetanide, 0.1 mM each, and brought to 37°C. K⁺Cl⁻ (with Rb⁺ as a tracer) was added to a final concentration of 5 mM. The reaction was stopped by washing four times in cold isotonic tris-buffered MgCl₂. The cells were lysed in distilled H₂O containing 0.1% Triton X100 and the proteins precipitated in 2.5% trichloracetic acid. Radioactivity was counted by the Cerenkov effect (see [36]). The fraction labelled ‘Na⁺K⁺ pump’ is inhibited ouabain, and ‘Na⁺K⁺Cl⁻ co-transport’ is inhibited by bumetanide.

The structure and function of this protein had been hitherto unknown, although the absence of the protein in association with the cation leak immediately suggested that it may have a role in cation transport, as a ‘plug’ rather than a standard channel, since it is missing in the cells with high transport rates.

Band 7 membrane protein
We purified this protein and obtained 43 amino acid residues of sequence by Edman degradation. From this knowledge, we prepared two redundant oligonucleotide primers, which enables us to amplify from a human bone marrow cDNA library a fragment of DNA of the appropriate size, given the separation of the primers on the amino-acid sequence [11]. This DNA fragment was then used to screen the same library, giving positively reacting clones. The open reading frame which contains DNA sequence coding for the Edman-determined amino-acids predicts a polypeptide of 31 kDa. An essentially identical sequence was arrived at independently by another group [18]. The hydropathy plot shows a single hydrophobic domain 29 amino acids long, quite close to the N-terminus, which can account for the integral nature of the protein. Both consideration of the electrostatic charge of the residues flanking the hydrophobic sequence and proteolytic digestion studies are consistent with the assertion that the long C-terminal domain is cytoplasmic [11, 19]. Database searches reveal no significant homology to other proteins and the sequence tells us little else of the structure and function of the protein. Northern blots show hybridization signals at 3000 bps (the same size as the cDNA) in RNA purified from a variety of sources, including human bone marrow and reticulocytes, liver, kidney, brain, HL60 cells and HeLa cells.

Biochemical studies have shown that the protein is phosphorylated and palmitoylated [20]. There is no para- amino salicylic acid staining band at 31 kDa on Laemmli gels [21]; there are no N-glycosylation sites on our putative extracellular sequence. Nearest neighbour analysis shows preliminary evidence that it binds to two proteins: itself and an entity of approximately 25 kDa, which could be ‘calpromotin’, a largely cytoplasmic protein which has been implicated in the process of activation of the Caz⁺-dependent K⁺ (Gardos) channel [22], but these studies require confirmation.

Genetic defect
Quite apart from the function of the Band 7 protein, the genetic defect which underlies the phenotype is likewise enigmatic. The condition is clearly inherited as an autosomal dominant, and is therefore presumably heterozygous: and yet, by Coomassie and silver staining and by Western blotting, it is clear that there is effectively no Band 7 protein present in the membranes of these patients. Messenger RNA is present in the reticulocytes, excluding a transcription abnormality. Exhaustive sequencing of multiple subclones reverse-transcribed and PCR-amplified from patient mRNA shows no abnormality in the coding region, although we have not yet excluded an abnormality in the 3' untranslated region.

It now seems that the lesion may lie either in another protein in the membrane, which may act as a ‘receptor’, or in post-translational modification. Abnormal phosphorylation of the τ protein has been suggested as a causative defect in cases of
Alzheimer's disease not attributable to any amyloid precursor t-protein defect [23]. While phosphorylation of t in Alzheimer's leads to the deposition of insoluble protein [24], phosphorylation can also target proteins for destruction [25]; a mutant kinase, making an error of commission in phosphorylating all band present phenotype. The hypothesis is testable in the premature degradation, would neatly explain the making an error of commission in phosphorylating Alzheimer's disease not attributable to any amyloid this, and the protein's nearest neighbours, to try to solve the problem.

Target proteins for destruction [26]; a mutant kinase, the hereditary stomatocytosis jigsaw has three conclusion parts: a leak to Na+ and K+, a missing protein of in a heterozygous disorder. We can speculate that the genetic defect does not lie in the stomatin gene, it could be that the putative channel is abnormal (and leaky), such that while the band 7 peptide is translated, it cannot be incorporated into the membrane and is degraded.

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Inducible transport systems in the regulation of parasite growth in malaria-infected red blood cells
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Introduction
Falciparum malaria remains the most prevalent human parasitic disease infecting over 100 million individuals each year [1, 2]. In an infected individual, parasitized red blood cells (PRBCs) containing young ring-form trophozoites of Plasmodium falciparum circulate freely and are relatively quiescent metabolically. As these asexual ring-forms mature, the host cells are removed from the circulation by adhering to post-capillary endothelial cells thereby sequestering in the microvasculature of many organs including the brain [3]. The pathophysiology of the disease is associated with the maturation of sequestered parasites and the subsequent release and re-invasion of merozoites at schizogony [1, 3]. During the course of an infection, the proportion of parasitized erythrocytes may reach 10–30% of the total, constituting 0.25–0.75 × 10¹⁰ metabolically active red cells. This is equivalent to a total volume of 500 ml PRBCs, which inter alia consume glucose and generate lactate at a rate that is one to two orders of magnitude higher than the rates in uninfected erythrocytes [4, 5]. Hypoglycaemia and lactate acidosis are common features of malaria [2, 3, 6].

Potential rate limitations of the host red cell membrane
In addition to their enhanced glycolytic rate, PRBCs take up and utilize a wide range of other important metabolic substrates, which normal mature uninfected red cells do not transport or utilize beyond the reticulocyte stage. Many exogenous amino acids are taken up and used in the synthesis of proteins by the parasite and are not derived, as is often assumed, from the proteolytic digestion of haemoglobin in the parasite’s food vacuole. Normal human erythrocytes, however, lack certain transport systems (one for the amino acid L-glutamate for example) and are almost impermeable to the closely related keto-acid 2-oxoglutarate. Therefore, unless modified during parasite development, the permeability of the host red cell membrane would be rate-limiting for the uptake of these substrates [7]. In the malaria-infected human red cell, glucose transport is unlikely to be rate-limiting when compared with the enhanced consumption of glucose drawn into the parasite’s glycolytic pathway. By contrast, this is not the situation in the murine malarias in which the constitutive influx rates in uninfected rodent red cells are approximately three orders of magnitude lower than those in human erythrocytes.

Changes in the surface properties of infected red cells during parasite maturation
From approximately 10–20 hours post-invasion, concomitant with cell surface antigenic changes and sequestration, the permeability characteristics of the host erythrocyte membrane are also modified. However, even after this developmentally regulated transformation of the transport properties, the influx rate for L-glutamate in the infected red cell remains less than 1% of that of L-glutamine [7]. Even though some L-glutamate could be derived from the digestion of haemoglobin, it is probable that exogenously derived L-glutamine is the precursor of much of the parasite’s requirement for L-glutamate, which is central to many reactions including the synthesis of nucleic acids, proteins [4] and glutathione [7].

Paucity of basic biochemical data relating to human malaria
Following the advent of continuous culture of P. falciparum [8, 9], it has been established empirically that adequate exogenous concentrations of both D-glucose and L-glutamine [7, 10] are essential for the