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THE RECONSTITUTION OF THE HUMAN ERYTHROCYTE SUGAR TRANSPORTER IN PLANAR BILAYER MEMBRANES

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The degradation of human erythrocyte membrane proteins in relation to the identification of the monosaccharide transporter has been investigated in whole membrane preparations and membrane protein extracts by polyacrylamide gel electrophoresis in sodium *n*-dodecyl sulphate and iodine-125 labelling. Evidence is presented for the degradation of band 3 polypeptide to lower molecular weight material some of which appears in region 4.5 of the polyacrylamide gel electrophoresis profile. It is found that the degradation process is inhibited by phenylmethylsulphonyl fluoride and is only significant in membrane extracts in the absence of detergent (Triton X-100) and on prolonged incubation at 37°C, conditions which do not prevail during the isolation of membrane protein extracts for reconstitution studies. Extracts of band 3 and band 4.5 have been prepared and reconstituted in bilayer lipid membranes. The permeabilities of the reconstituted systems to D-glucose have been investigated and it is found that only bilayers incorporating band 4.5 exhibited enhanced monosaccharide transport. A linear relationship between D-glucose transport and the concentration of protein in the aqueous phase bathing the bilayers suggests a partitioning of the protein into the bilayer. Reconstitution is stereospecific and inhibited by cytochalasin B.

Introduction

The stereospecific monosaccharide transport activity of the human erythrocyte membrane can be reconstituted in liposomes and planar bilayer lipid membranes by isolation and incorporation of the sugar transporter [1]. There is increasing evidence that the transporter is a transmembrane glycoprotein of molecular weight approx. 55000 which can be identified by polyacrylamide gel electrophoresis at the high molecular weight end of the so-called region 4.5 of the gel profile [1,2]. This identification is supported by reconstitution studies in bilayers [3] and liposomes [4] and on the basis of cytochalasin B binding [5,6] and immunological evidence [7,8]. However, it has been argued that the association between the region 4.5 polypeptide and sugar transport is artifactual and arises as a consequence of the proteolysis of band 3, the major transmembrane polypeptide of molecular weight approx. 95000 [3,9–11]. Although around 90% of the band 3 polypeptides are believed to be associated with anion transport there are enough copies of the residual 10% to account for the approximately 10^5 sugar transport sites per cell [12].

In this paper we describe experiments to ascertain the conditions under which proteolysis of band 3 can occur, the isolation and characteristics of the sugar transporter free of cross-contamination with band 3 or its degradation products and the incorporation of band 3 and the sugar transporter in planar bilayer lipid membranes.

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; EDTA, ethylenediamine tetraacetate.

Materials and Methods

Preparation of erythrocyte ghosts and Triton extracts and degradation procedures. Haemoglobin-free ghosts were prepared from phosphate buffered saline (buffer A) washed human erythrocytes from out-dated transfusion blood by hypotonic lysis according to the method of Dodge et al. [13]. The membranes were extracted at a final protein concentration of 1.0 to 1.5 mg · ml^{-1} by mixing 1 vol. of ghosts with 5 vol. of 40 mM Tris-HCl (pH 8.0)/0.5% Triton X-100 [14] for 20 min at 0°C followed by centrifugation at 77000 × *g* or 100000 × *g* for 40 min at 4°C. The supernatant was designated as a '40T' extract. Protein and Triton X-100 in the extracts were assayed by the methods of Wang and Smith [15] and Garewal [16], respectively. Protease activity was inhibited by incubation of the membranes and membrane extracts with phenylmethylsulphonyl fluoride (PMSF) at a concentration of 0.1 g/g membrane protein using an ethanolic stock solution of PMSF. Calcium chloride was added to the buffers to a concentration of 1 mM.

Degradation was assessed from gel profiles after incubation of samples of membranes and 40T extracts prepared in the absence of PMSF at 37°C for varying time periods both prior to and after removal of Triton X-100 by gel filtration using a Sephadex G50 column. Gel-electrophoresis was carried out by the method of Neville and Glossman [17] on 7.5 or 10% polyacrylamide slabs or cylindrical gels. Gels were scanned at 550 nm on a Beckman Acta spectrophotometer. Gel profiles were quantified by weighing sections cut from photocopied chart paper scans.

Preparation of iodinated ghosts and Triton extracts and degradation procedures. Phosphate-buffered saline washed erythrocytes were iodinated by the lactoperoxidase-glucose oxidase-Na^{125}I method [18]. The washed cells were suspended to a haematocrit of 20% and incubated for 45 min at 4°C in buffer A containing 0.3 μM lactoperoxidase, glucose oxidase (50 mU · ml^{-1}), 2 mM glucose and Na^{125}I (1 mCi · ml^{-1}). The iodination reaction was stopped by washing 3-times with buffer A. Iodinated ghosts were prepared by hypotonic lysis [13] to a final protein concentration of 6 mg/ml and had an activity of (2.8–5) · 10^8 cpm/g of protein. The protein gel profiles and ^{125}I-labelling pattern of the ghosts were assessed by electrophoresis and γ-counting as described below. Membrane extracts (40T extracts) were prepared from isotopically diluted ghosts (1 volume of ^{125}I-labelled ghosts to 3 volumes of unlabelled ghosts) as described above.

Degradation of ^{125}I-labelled 40T extracts was assessed by gel electrophoresis [17] on 10% slab gels. The slabs were scanned spectrophotometrically and then cut into 50 slices for radioactive γ-counting. Degradation of the extracts prior to and after removal of Triton X-100 by gel filtration using a Sephadex G50 column was monitored after 60 min incubation at 37°C and terminated by immersion in liquid nitrogen.

Preparation of band 3 and band 4.5 polypeptide extracts. Haemoglobin-free erythrocyte membranes were prepared by hypotonic lysis and incubated with 0.1% (w/v) PMSF. Extrinsic proteins were removed from the membranes by incubation twice with 7 volumes of 0.1 mM EDTA [19] and intrinsic proteins were eluted with 3 volumes of 0.67% (w/v) Triton X-100 in 10 mM Tris-HCl buffer pH 8 [14]. The intrinsic proteins were fractionated by ion-exchange chromatography on a Whatman DEAE-cellulose DE-52 column. An unbound fraction (I) of region 4.5 polypeptide was eluted at low ionic strength with 40 or 50 mM Tris-HCl buffer (pH 8), containing 50 mM NaCl and 0.5% (w/v) Triton X-100, and a fraction (II), predominantly band 3, was eluted at high ionic strength with the Tris-HCl buffer containing 500 mM NaCl and 0.5% (w/v) Triton X-100 [2,20]. Triton X-100 was removed from the fractions by incubation overnight with Biobeads SM-2 (0.4 g · cm^{-3} plus 0.05% (w/v) PMSF). For fraction I dipalmitoylphosphatidylcholine (0.025 mg · cm^{-3}) was added during incubation to increase protein recovery and it was then concentrated by dialysis against sucrose and dialysed against 40 mM Tris-HCl buffer (pH 8). Both protein fractions were frozen as droplets in liquid nitrogen and stored at −25°C.

Permeability measurements. The unidirectional flux of radioactively labelled sugars (180–200 μM) across the bilayers was measured using procedures and a similar apparatus as previously described [3,21]. Permeability coefficients (*P*) were de-

termined from plots based on the equation

$$C_oV_o/C_iA = Pt \quad (1)$$

where C_o is the concentration of sugar on the *trans* side of the bilayer of area A in contact with an aqueous phase of volume V_o and C_i is the concentration of sugar on the *cis* side of the bilayer from which the radiolabelled sugar is diffusing. The bilayers were formed from equimolar solutions of chromatographically pure egg lecithin and cholesterol in *n*-decane [7] and stored under nitrogen at −20°C. Before use excess Triton X-100 was removed by passing the extracts down a Sephadex G50 column. The extracts were added to the balanced salt solution bathing the *trans* side of the membrane.

Amino acid analysis. Protein extracts were hydrolysed in 6 M HCl for 24 h at 105°C and the amino acid composition was determined with a JEOL 6AH analyser.

Results

Degradation studies

The degradation of membrane polypeptides in erythrocyte ghosts was found to occur slowly at 4°C but increased significantly on prolonged incubation at 37°C. An example of the changes in the gel profiles of ghosts after incubation of the ghosts in hypotonic saline for 2 h at 37°C is shown in Figs. 1a and 1b. There is a significant increase in the amount of material in region 4.5 largely at the expense of band 3. These and similar gel profiles were used to draw Fig. 1c where the changes in relative absorbance at 550 nm for the band 3 peak and the major peak in region 4.5 are shown as a function of time. To construct Fig. 1c the gel profiles were aligned and the ΔA_{550} readings for intervals along the R_m axis were measured. PMSF was effective in inhibiting the degradation at 37°C (Fig. 1c).

The degradation of Triton X-100 extracts of ghosts was investigated by electrophoresis both before and after removal of Triton X-100 by gel filtration. Table I shows that the Triton to protein weight ratio in the extracts was in the ranges 5.8 to 7.5 for several membrane preparations from different blood batches and extractions. Gel filtration removed almost all the Triton and gave turbid suspensions which contained largely protein and some membrane lipids (see below). The gel profile of extracted polypeptides is maintained on rapid freezing or storage at 4°C for short time periods (less than 24 h) and on incubation at 37°C for upto 1 h. On removal of Triton X-100 after 1 h incubation at 37°C the gel profile changed significantly (compare Figs. 2a and 2b). Again there was an increase in region 4.5 polypeptide and a concomitant loss of band 3. A difference profile is shown in Fig. 2c and the time dependence of the degradation is shown in Fig. 2d.

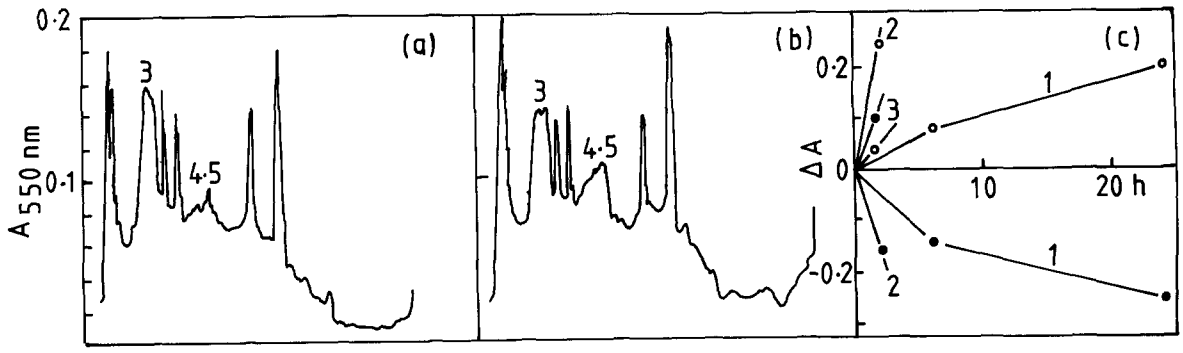

Fig. 1. Degradation of membrane polypeptides in erythrocyte ghosts. (a) Gel profile of erythrocyte membrane polypeptides. The ghosts were prepared by hypotonic lysis in saline phosphate and incubated at 4°C for 15 min prior to electrophoresis. (b) Gel profile after incubation of the ghosts in hypotonic saline phosphate at 37°C for 2 h prior to electrophoresis. (c) Relative absorbance changes (ΔA_{550nm}) of band 3 (●) and band 4.5 (○) 'peak heights' as a function of time of incubation at 4°C (curves 1), 37°C (curves 2) and 37°C with addition of PMSF (curves 3).

TABLE I

REMOVAL OF TRITON X-100 FROM '40T' EXTRACTS BY GEL FILTRATION (SEPHADEX G50)

Concentrations are given in μg/ml. n.d., not detectable.

Batch (extract)	Pre-filtration			Post-filtration	
	[Protein]	[Triton]	[Triton]/[Protein]	[Protein]	[Triton]
A(1)	810±25	5000	6.2	240±15	<10.
B(1)	670±60	5000	7.5	530±40	50±20
B(2)	860±27	5000	5.8	310±20	30±10
C(1)	710±38	5000	7.0	480±56	n.d.
C(2)	860±40	5000	5.8	325±85	n.d.
D	725±20	5000	6.9	690±30	25

The results suggest that degradation arose as a consequence of endogenous proteinase activity inhibited by PMSF and Triton X-100. To investigate the possibility that the proteinase activity arose from lymphocytes during blood storage extracts were prepared from fresh blood cells from which lymphocytes had been removed by filtration during donation. Degradation was observed in extracts prepared from these cells on removal of Triton X-100. In general we found that while degradation was always observed the extent of degradation varied from extract to extract rather than from one batch of ghosts to another.

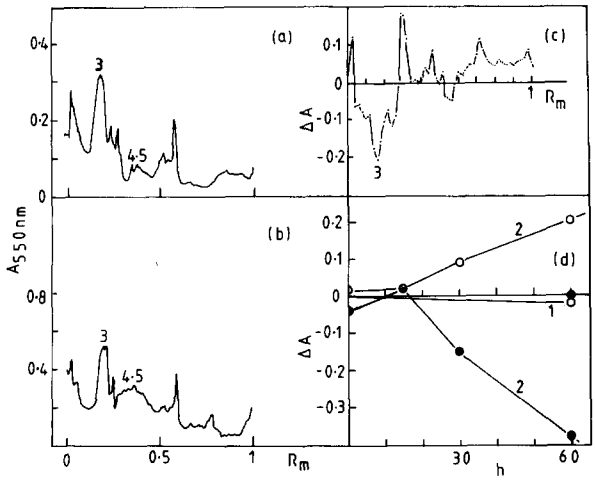

Fig. 2. Degradation of Triton X-100 ('40T') extracts of erythrocyte ghosts. (a) Gel profile of Triton X-100 (40 mM Tris-HCl, pH 8) extract after incubation at 37°C for 60 min. (b) Gel profile of Triton X-100 extract after removal of excess Triton X-100 by gel filtration (Sephadex G50) and incubation at 37°C for 60 min. (c) Difference gel profile, $\Delta A = A$(post-gel filtration) − A(pre-gel filtration). (d) Relative absorbance changes (ΔA_{550}) of band 3 (●) and band 4.5 (○) 'peak heights' as a function of time of incubation. Curves 1 in the presence of excess Triton X-100, curves 2 after gel filtration to remove excess Triton X-100.

Degradation was investigated in Triton X-100 extracts from ^{125}I-labelled ghosts. Figs. 3a and b show gel profiles and labelling patterns for ghosts and '40T' Triton X-100 extracts. Both band 3 and region 4.5 polypeptides are labelled. Removal of Triton X-100 by gel filtration followed by incubation for 1 h at 37°C results in loss of labelled polypeptide from the band 3 region and an increase of labelled material in region 4.5 and also much lower molecular weight material with relative mobility greater than 0.9. Table II shows the distribution of labelled polypeptide in ghosts and extracts prior to and after removal of Triton X-100 and incubation at 37°C. The results support the proposition that degradation of band 3 polypeptides in the absence of PMSF occurs on extraction only when Triton X-100 is removed and at elevated temperatures. The removal of detergent alone appears to produce only minimal changes on storage at 4°C for 12–18 h.

Isolation of the sugar transporter

During the course of this work 21 experiments to isolate the transporter were carried out. A number of variations in the procedure described above (Materials and Methods) were made in attempts to investigate changes in experimental conditions on

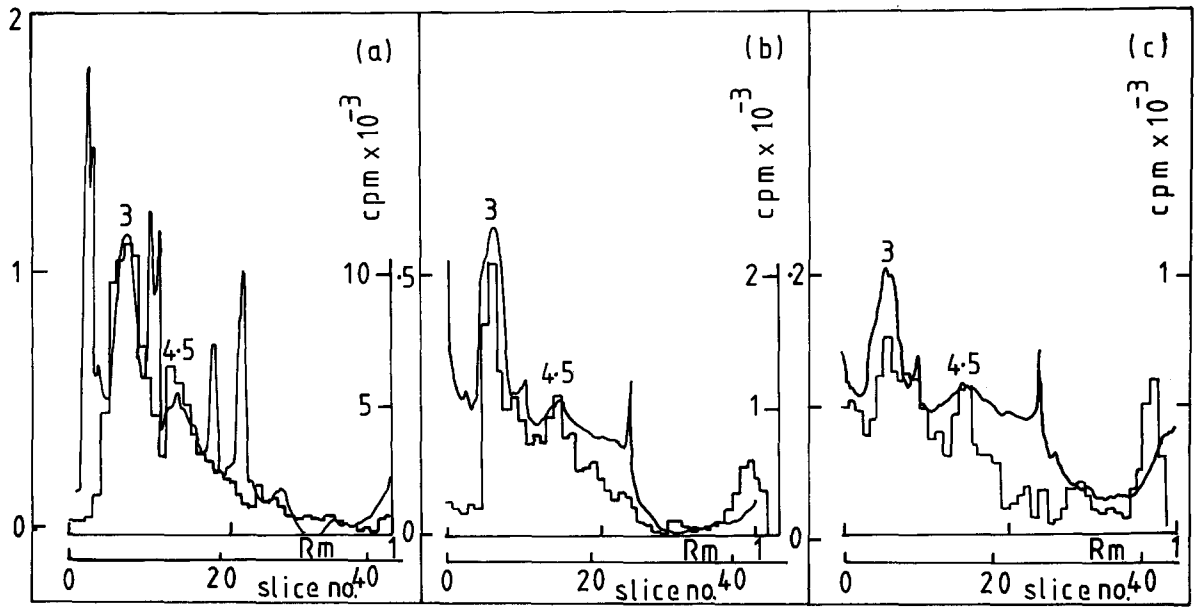

Fig. 3. ^{125}I-labelling of erythrocyte ghosts and Triton X-100 extracts of membrane proteins. (a) Gel profile and ^{125}I-labelling pattern (histogram and right hand axis) of erythrocyte ghosts. (b) Gel profile and ^{125}I-labelling pattern of Triton X-100 (40 mM Tris-HCl, pH 8) extract of erythrocyte membrane proteins. (c) Gel profile and ^{125}I-labelling pattern of Triton X-100 extract after removal of excess Triton by gel filtration (Sephadex G50) and incubation for 60 min at 37°C.

the effectiveness of ion-exchange in the separation of band 4.5 (the transporter) from the major contaminant (band 3). In principle isolation of the transporter is done by application of Triton X-100 solubilized extracts of band 3 and the transporter in low ionic strength media to the ion-exchange column followed by elution at low ionic strength to give a fraction I (the transporter). Subsequent elution at higher ionic strength gives a fraction II (largely band 3). Fig. 4 shows an example of a separation using gradient elution. In this experiment a total of 13 mg of protein was applied to the column and fractions I and II contained 786 μg (6.1% of the total protein applied) and 4312 μg (33%), respectively. Very similar results were obtained by step-wise elution of band 3 using 40 mM Tris-HCl (pH 8) plus 0.5 M NaCl. The ionic strength of the medium in which the extracts were applied to the column had a slight effect on the relative amounts of protein in fractions I and II.

TABLE II

DISTRIBUTION OF ^{125}I-LABELLED MATERIAL IN GHOSTS AND TRITON X-100 EXTRACTS OF GHOSTS

System	% label			Ratio % label 3/ % label 4.5
	in band 3	in region 4.5	with $R_m > 0.9$	
Ghosts (1)	46	14	6.8	3.3
Ghosts (2)	50	19	5.6	2.6
'40T' extract	42	19	11	2.2
'40T' extract (after 1 h at 37°C)	34	19	8.7	1.8
'40T' extract (−Triton)	35	17	9.5	2.1
'40T' extract (−Triton after 1 h at 37°C)	30	21	8.7	1.4

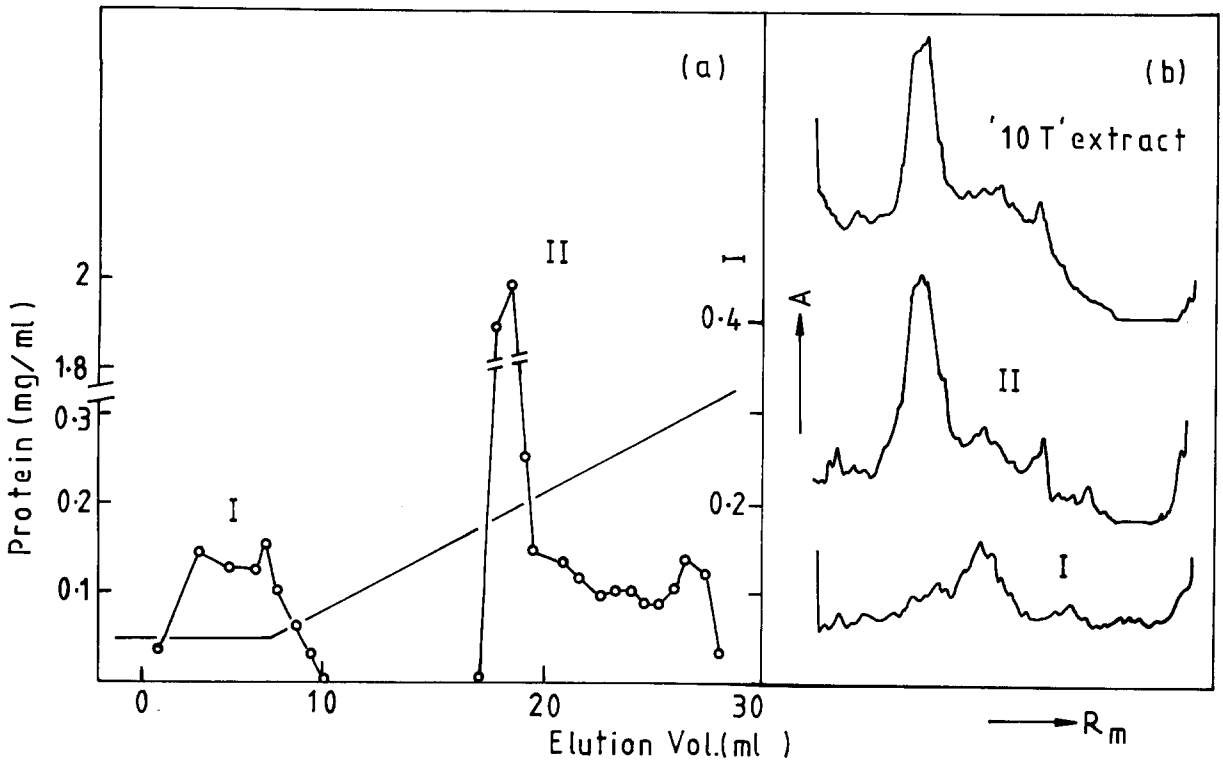

Fig. 4. (a) Separation of band 3 (fraction II) from band 4.5 (fraction I, the sugar transporter) as their Triton X-100 complexes by gradient elution on a DEAE ion-exchange column. Line without points is the ionic strength gradient (right hand axis). (b) Gel profiles of Triton X-100 extract (10 mM Tris-HCl, pH 8) applied to the ion-exchange column and fractions I and II.

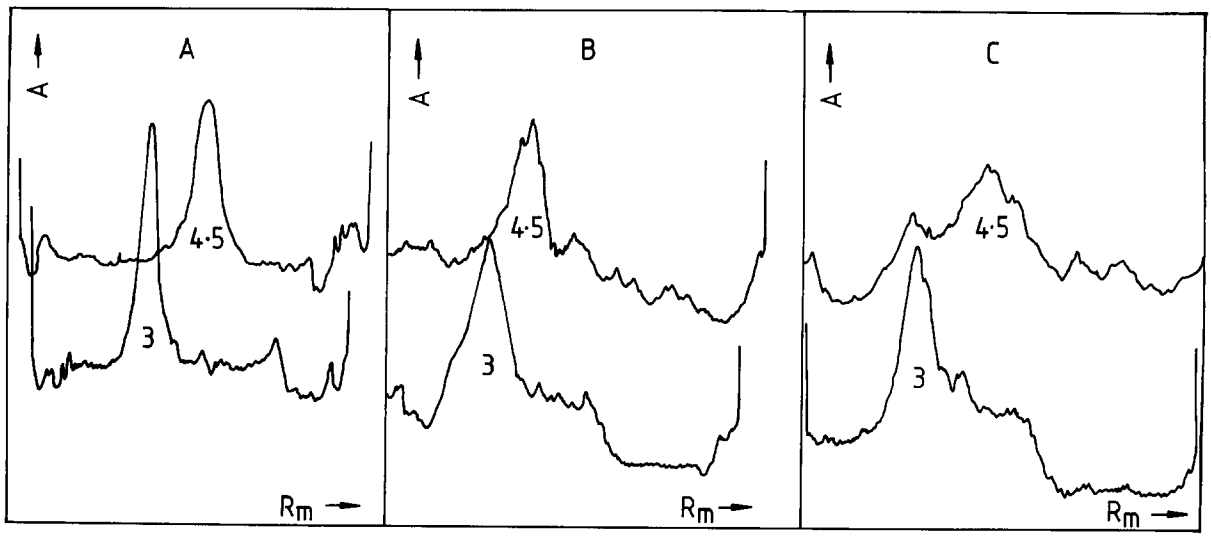

Fig. 5. Gel profiles of band 3 (lower traces) and band 4.5 (the sugar transporter) from three independent isolations (A, B and C).

TABLE III

AMINO ACID COMPOSITION OF MEMBRANE PROTEINS (mol%)

Amino acid	4.5	4.5 (Ref. 2)	3	3 (Ref. 23)
Basic				
Lys	3.8	3.6	4.0	3.3
His	2.1	1.2	2.5	1.9
Arg	5.9	4.1	4.5	5.4
Total	11.8	8.9	11.0	10.6
Acidic				
Asp	5.4	5.4	6.6	6.8
Glu	10.0	9.7	9.9	12.4
Total	15.4	15.1	16.5	19.2
Neutral				
Thr	5.7	5.4	7.1	4.9
Ser	9.5	7.6	8.0	6.4
Pro	1.6	4.8	5.8	6.7
Gly	12.6	9.4	7.6	7.5
Ala	8.9	8.0	7.8	7.2
Cys				
Total	38.3	35.2	36.3	32.7
Hydrophobic				
Val	5.4	8.6	8.0	7.4
Met	3.9	2.1	2.0	2.2
Ile	5.1	6.9	6.1	4.6
Leu	12.6	11.1	11.7	15.2
Tyr	3.3	2.7	3.2	2.4
Phe	4.1	7.3	5.0	5.6
Total	34.4	38.7	36.0	37.4

Application in 10 mM Tris-HCl (pH 7.5) gave weight ratios (I/II) in the range 0.04–0.25 (three isolations) whereas application in 50 mM Tris-HCl (pH 7.5) gave weight ratios (I/II) in the range 0.20–0.52 (four isolations). Variations were also made in the ratio of sample to ion-exchange column volume from 10 to 40 without significantly changing the ratio (I/II) or the cleaness of the separations. Introduction of sonication steps prior to ion-exchange (12 cycles 10-s periods on-off at 10 μm amplitude with a MSE Soniprep 150 followed by 20–30 min in a bath sonicator on ice) made no significant difference to the purity of the isolated transporter.

Permeability measurements were made on 3 transporter isolates designated as samples A, B and C whose gel profile are shown in Fig. 5. Sample A had the highest transport activity (see below) and its amino acid composition and that of the corresponding band 3 preparation are compared with literature data in Table III.

Permeability measurements

Table IV shows the protein to lipid and Triton to protein weight ratios for the transporter (fraction I) and band 3 (fraction II) extracts from three isolations (A, B and C) and their effects on the D-glucose permeabilities of planar phosphatidylcholine-cholesterol bilayers when present at similar concentrations in the solution bathing the *trans* side of the bilayer. The final column shows the enhancement of permeability (P_{rel}) relative to the passive permeability of D-glucose through the bilayer in the absence of protein. Under these conditions band 3 does not significantly enhance permeability to D-glucose. Since sample A gave the largest enhancement effect most permeability measurements were made with this sample. Fig. 6 shows

TABLE IV

EXTRACT COMPOSITION AND D-GLUCOSE TRANSPORT ACTIVITY (PERMEABILITY) OF SUGAR-TRANSPORTER AND BAND 3 ON INCORPORATION INTO BILAYER LIPID MEMBRANES

Sample	Protein/lipid ratio (w/w)	Triton/protein ratio (w/w(	Protein ($\mu g \cdot cm^{-3}$)	P ($cm \cdot s^{-1}$) ($\times 10^8$)	P_{rel} (P/P_0) [a]
A Fraction I	1.26	<0.05	1.04	46.4±0.8	8.5±0.2
A Fraction II	2.92	<0.006	1.29	8.3±0.6	1.6±0.1
B Fraction I	0.57	<0.13	1.01	17.9±0.8	3.3±0.4
B Fraction II	3.00	0.04	1.54	6.4±1.2	1.2±0.2
C Fraction I	n.d. [b]	<0.19	1.05	16.7±0.8	3.0±0.4

[a] $P_0 = (5.49 \pm 2.89) \cdot 10^{-8}$ $cm \cdot s^{-1}$.

[b] n.d., not determined.

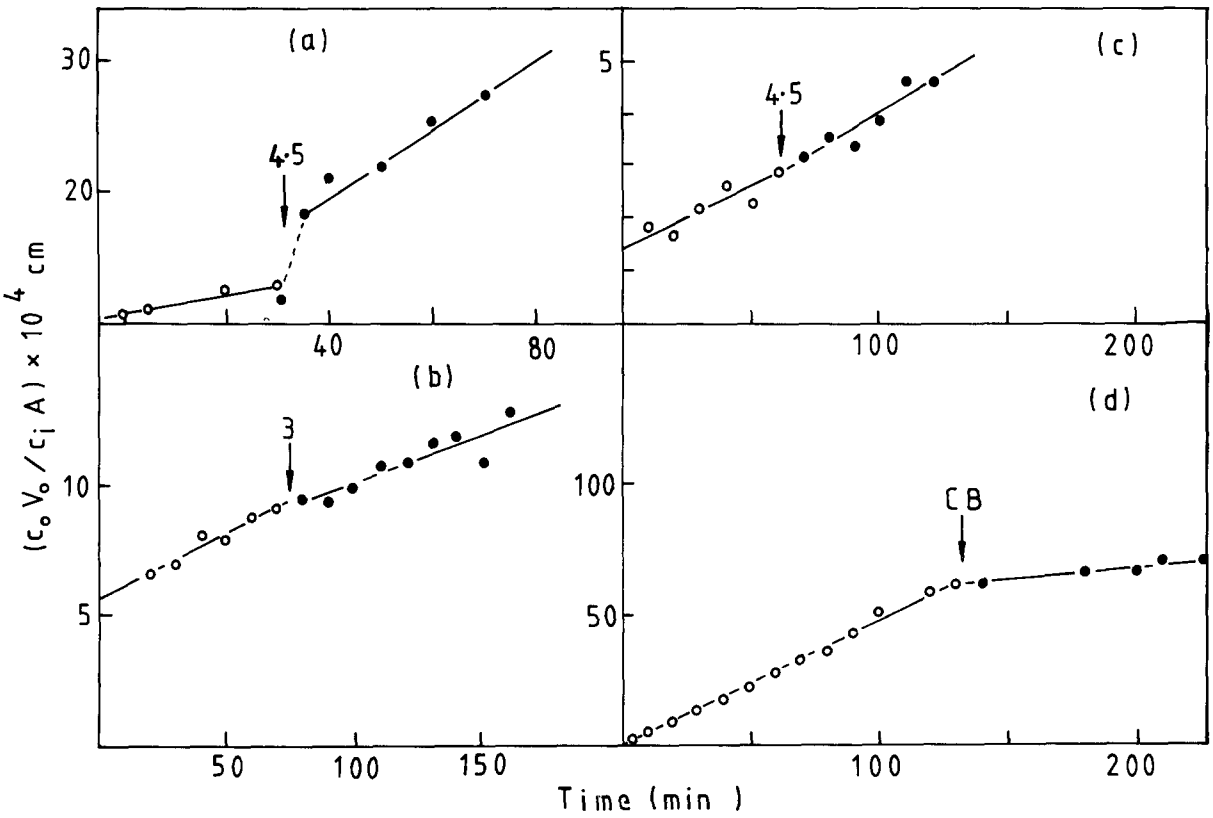

Fig. 6. Sugar permeability plots for bilayer lipid membranes (egg lecithin-cholesterol in *n*-decane) at 25°C. The plots are based on Eqn. 1. The slopes of the plots are equal to the permeability coefficients. (a) Passive diffusion of D-[^{14}C]glucose (○) and facilitated diffusion (●) on addition of band 4.5 (sugar transporter) at a concentration of 0.99 $\mu g \cdot cm^{-3}$ to the *trans* side of the bilayer. (b) Passive diffusion of D-[^{14}C]glucose (○) and diffusion (●) on addition of band 3 at a concentration of 1.54 $\mu g \cdot cm^{-3}$ to the *trans* side of the bilayer. (c) Passive diffusion of L-[^{14}C]glucose (○) and diffusion (●) on addition of band 4.5 (sugar transporter) at a concentration of 1.01 $\mu g \cdot cm^{-3}$ to the *trans* side of the bilayer. (d) Facilitated diffusion of D-[^{14}C]glucose (○) with band 4.5 (sugar transporter) at a concentration of 1.25 $\mu g \cdot cm^{-3}$ on the *trans* side of the bilayer and on addition of cytochalasin B (26 μM) to the *trans* side (●).

some representative plots based on Eqn. 1 from which permeability coefficients were derived. Figs. 6a and 6b show the effects of the addition of transporter and band 3 extracts to the bilayer system. Addition of protein (either transporter or band 3) to the bilayer system can give rise to a transient increase in permeability (e.g. as in Fig. 6a). An initial higher transport rate was also sometimes found in passive permeability measurements. This effect occurred in approx. 25% of the experiments (as depicted in Fig. 6) and as it was not specific to protein addition probably arises from mechanical disturbance of the bilayer. Band 3 incorporation does not significantly change the bilayer permeability in marked contrast to the transporter. The stereospecificity of the reconstituted system is seen by comparing Figs. 6a and 6c for D- and L-glucose transport, respectively. The passive permeability of L-glucose was found to be 4.39 $(\pm 0.73) \cdot 10^{-8}$ cm^{-1} from several independent experiments. The inhibition of transport by cytochalasin B is illustrated in Fig. 6d. In a control experiment we found that cytochalasin had no effect on the passive D-glucose permeability of the bilayer.

The effect of the concentration of the transporter extract on transport enhancement is shown in Fig. 7. The relative permeability was found to increase linearly with protein concentration in the bathing solution. Band 3 was found to have no

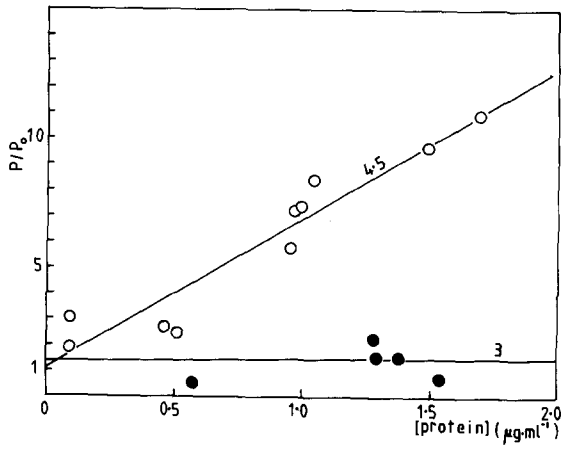

Fig. 7. Relative permeability (P/P_0) of D-glucose at 25°C as a function of protein concentration in the aqueous phase for bilayers incorporation band 3 (●) and band 4.5 (sugar transporter) extracts (○).

significant effect on D-glucose permeability upto a protein concentration of 8.2 $\mu g \cdot cm^{-3}$ (not shown). In previous reconstitution studies with bilayers using less pure membrane extracts we failed to establish a definite relationship between permeability enhancement and protein concentration in the aqueous phase [3,21].

Discussion

The identification of the human erythrocyte sugar transporter has proved to be a controversial problem [1]. There is substantial evidence that band 4.5 is the active transporter [2–8]. Batt et al. [24] were amongst the first to propose this identification on the basis of differential labelling studies with the impermeable glutathione-maleimide and further support subsequently came from cytochalasin B binding studies [5,6], immunological experiments [7,8] and reconstitutions in liposomes [4,25] and bilayers [3]. More recently, however, Mullins and Langdon [9,10] prepared an affinity label (maltosyl isothiocyanate) for the transporter, experiments with which led them to the view that the transporter was a component of band 3 and that the assignment to band 4.5 was artifactual and arose as a result of proteolysis of band 3 as had been previously suggested [3]. Experiments (gel filtration and reconstitution in liposomes) with cholate extracts also point to a component of band 3 as the transporter [26,27].

On the basis of the degradation studies described above we can find little support for significant proteolytic degradation of band 3 in Triton X-100 extracts in the presence of excess detergent and PMSF. In our hands, band 3 degradation only occurs in the absence of detergent and only then after prolongeod incubation at 37°C; conditions which do not prevail during the isolation of the extracts for the permeability studies.

It is clearly difficult to achieve clean separations of band 3 and 4.5 protein (Fig. 5). We have explored the effects of various modifications in the conditions for the separation by ion-exchange chromatography but have had only limited success in developing a totally reliable and reproducible procedure. Amino acid analysis (Table III) shows that in terms of overall hydrophobic to hydrophilic balance both band 3 and 4.5 are very similar; taking note of the fact that the protein extracts are solubilized as protein-lipid-Triton complexes the separation by ion exchange may depend on the different extents of glycosylation of the proteins.

The results in Figs. 6 and 7 show that reconstitution studies with the cleanest extracts of band 3 and 4.5 show that under these conditions transport activity is only reconstituted with band 4.5 and band 3 does not transport D-glucose. We cannot however completely exclude the possibility that the band 3 extracts do not partition into the bilayers and/or are in a denatured (inactive) state. This result supports much previous work [2–8,24,25] but is in conflict with the work of Mullins and Langdon [9,10] and to some degree with that of Lundahl et al. [26,27].There appears to us to be no simple explanation for the conflict between the results obtained by Mullins and Langdon, the present results and those of the other workers. Although Lundahl et al. [26,27] give preference to the association between glucose transport and a sub-band of 3, their molecular sieve chromatographic separations of cholate extracts give relatively wide distributions of solubilized protein complexes and the stereospecific transport of the most active liposomes prepared from these extracts can only be partly attributed to band 3 as they also contain region 4.5 polypeptides.

The linear relationship (Fig. 7) between transport activity (as expressed by P/P_0) and protein concentration in the aqueous phase suggests a

partition of protein into the bilayer. The mechanism of the partitioning process is not known but preliminary studies by electron microscopy show that after passage down a gel column Sephadex (G50) to remove excess Triton X-100 (as required prior to reconstitution in planar bilayers) the protein-lipid extracts are solubilized in the form of vesicles with diameters in the range 30–170 nm. Partitioning may thus occur by vesicle-bilayer fusion. The increase in P/P_0 with protein concentration implies that under the conditions used here saturation of the bilayer with transporters does not occur. These results suggest that it would be possible to reconstitute the transporter quantitatively in a bilayer by measurement of the number of fusion events between vesicles of known transporter content and a planar bilayer.

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