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THE PERMEABILITY OF BILAYER LIPID MEMBRANES ON THE INCORPORATION OF ERYTHROCYTE MEMBRANE EXTRACTS AND THE IDENTIFICATION OF THE MONOSACCHARIDE TRANSPORT PROTEINS

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Summary

1. Extracts of the human erythrocyte membrane have been prepared by five different procedures involving Triton X-100 solubilization and gel chromatography.

2. The extracts have been analysed by gel electrophoresis and incorporated asymmetrically into phosphatidylcholine-cholesterol-*n*-decane planar bilayers.

3. Removal of excess Triton X-100 from membrane extracts or prolonged storage facilities the proteolysis of membrane extracts with the partial or complete breakdown of band 3 polypeptides (notation of Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2616) and the appearance of an enhanced zone 4.5 and low molecular weight material.

4. Incorporation of zone 4.5 polypeptides into bilayer lipid membranes increases their permeability to D-glucose at 27 and 5°C.

5. It is suggested that the components of the monosaccharide system are present in band 3 polypeptides but that they can undergo proteolysis with some retention of transport activity.

Introduction

The identification of the human erythrocyte membrane proteins involved in the facilitated diffusion of monosaccharides is presenting a considerable challenge. Several investigators have produced evidence for the involvement of the *trans* membrane polypeptides of band 3 of the electrophoretogram of the erythrocyte membrane proteins (notation of Fairbanks et al. [1]) in monosaccharide transport [2—7]. More recently, however, some of these workers [8,9] and others [10] have presented evidence that proteins giving rise to a broad band designated as zone 4.5 of the electrophoretogram are components

of the monosaccharide transport system. This zone centered around an apparent molecular weight of approx. 55 000 accounts for approx. 11% of the Coomassie Blue-stainable material of the membrane [11].

Our own studies in this field have been concentrated on attempts to incorporate the monosaccharide transport system into planar bilayer lipid membranes [12—15]. In a previous paper [15] a reconstituted system having a D-glucose permeability of magnitude comparable to that of the human erythrocyte membrane was described. It was suggested that the band 3 polypeptides were associated with monosaccharide transport. In this paper we describe the effect of several erythrocyte membrane extracts including those reported to contain predominantly band 4.5 polypeptides [8,9] on the D-glucose permeability of bilayer lipid membranes. The results indicate that while a facilitated transport system for D-glucose can be reconstituted in bilayers in the presence of membrane extracts containing predominantly zone 4.5 polypeptides and little or no band 3 species there are very strong indications that the lower molecular weight species present in zone 4.5 arise from proteolysis of band 3 components and may be the transmembrane fragments of band 3 which are responsible for facilitated transport of monosaccharides.

Experimental

Preparation of membrane extracts. All the membrane extracts were prepared from haemoglobin-free 'ghosts' obtained from outdated blood-bank blood by a modification of the method of Dodge et al. [16]. The extracts were analysed by SDS-polyacrylamide gel electrophoresis according to the procedure of Fairbanks et al. [1].

The gels were scanned at 580 nm on an Acta C III spectrophotometer. Protein contents of the extracts were measured by modified Lowry assays for detergent-containing extracts [17] and detergent-free extracts [18]. Phospholipid was assayed by the zinzade method [19] and Triton X-100 by the method of Garewal [20]. The following membrane extracts were prepared:

(1) An extract containing principally zone 4.5 according to the procedure of Kahlenberg and Zala [9]. Fresh frozen haemoglobin-free 'ghosts' (protein content approx. 3 mg · ml^{-1}) were warmed to 4°C and incubated with a solution containing 0.125% Triton X-100, ionic strength 0.02, pH 8.0 (solution 1 of Kahlenberg and Zala [9]) for 15 min. The volume ratio of ghost suspension to solution 1 was 0.25. The suspension was then centrifuged at $10^5 \times g$ for 1 h at 4°C and the supernatant was passed down a Whatman DEAE-cellulose DE 52 ion-exchange column. The column was then washed with a solution containing 0.1% Triton X-100, ionic strength 0.02, pH 8.0 (solution 2 [9]) and eluted with a solution containing 0.1% Triton X-100, ionic strength 0.1, pH 8.0 (solution 3 [9]) at a rate of 50 ml · h^{-1}. Fractions (5 ml) were collected and monitored for protein content.

(2) An extract containing principally zone 4.5 according to the procedure of Kasahara and Hinkle [5,8]. 1 vol. ghost suspension (protein content approx. 5 mg · ml^{-1}) was incubated with 10 vols. 0.1 mM EDTA, pH 8.0, for 20 min at 37°C, followed by centrifugation at 16 000 $\times g$ for 1 h at 4°C. The pellet was washed with the EDTA solution and centrifuged and then treated with 4 vols.

0.5 M NaCl, 5 mM Tris-H_2SO_4, pH 7.4, for 20 min at 4°C followed by centrifgation at 16 000 $\times g$ for 30 min at 4°C. The pellet was washed once with 2 vols. 10 mM Tris-H_2SO_4, pH 7.4. The pellet was extracted with 1.25 vols. 0.5% Triton X-100 in 10 mM Tris-H_2SO_4, pH 7.4, for 20 min at 4°C, centrifuged at $10^5 \times g$ for 1 h at 4°C and then the supernatant was passed down a DEAE cellulose column previously equilibrated with the 0.5% Triton X-100 solution. The column has eluted with a solution containing 0.5% Triton X-100, 50 mM Tris-HCl, 1 M NaCl, pH 7.4. Fractions (4 ml) were collected and monitored for protein content.

(3) A Triton X-100 extract of dimethyl maleic anhydride treated haemoglobin-free and spectrin-depleted 'ghosts'. Haemoglobin-free ghosts were successively washed with 0.1 mM EDTA, 0.1 M Tris-HCl, pH 8.0, and 8 mM Tris-HCl, pH 8.0, to remove spectrin. To the resulting ghost suspension (10 ml) containing approx. 20 mg protein plus 12 ml water was added 10 mg dimethyl maleic anhydride with continuous stirring over 40 min. The pH of the reaction mixture was maintained at 8.0 by the addition of 0.1 M NaOH [2]. The mixture was then centrifuged at 77 000 $\times g$ for 30 min at 4°C. The pellet was washed with 8 mM Tris-HCl, pH 8.0, and suspended in 8 mM Tris-HCl. The suspension (1 vol. containing approx. 2 mg · ml^{-1} protein) was incubated with 3 vol. 0.5% Triton X-100 in 8 mM Tris-HCl, pH 8.0, for 30 min at 4°C and centrifuged at $10^5 \times g$ for 1 h at 4°C. The required supernatant contained approx. 0.8 mg · ml^{-1} protein.

(4) A Triton X-100 '8T extract' prepared according to the procedure previously described [15] but containing cross-linked dimeric band 3 polypeptides. Spectrin-depleted 'ghosts' (5 ml, protein content 5 mg · cm^{-3}) were dispersed in 30 ml 8.5 mM phosphate buffer, pH 7.5, and centrifuged at 77 000 $\times g$ for 30 min at 4°C. The pellet was washed twice with 30 ml of the phosphate buffer and finally suspended in 15 ml buffer to which was added 15 ml buffer solution containing 100 μM *o*-phenatholine and 20 μM $CuSO_4$ [3]. The reaction mixture was incubated for 20 min at room temperature and then centrifuged at 77 000 $\times g$ for 30 min at 4°C. The pellet was washed with 10 mM Tris-H_2SO_4, pH 7.4, and suspended in 7.5 mM phosphate buffer, pH 7.5. 1 vol. suspension was incubated with 5 vols. 0.5% Triton X-100 in 8 mM Tris-HCl, pH 8.0, for 20 min at 0°C followed by centrifugation at $10^5 \times g$ for 1 h at 4°C. The required supernatant contained approx. 0.9 mg · ml^{-1} protein.

(5) A Triton X-100 '8T extract' prepared as previously described [15] and then passed down a DEAE-cellulose column and eluted with 0.5% (w/v) Triton X-100, 8 mM Tris-HCl, pH 8.0, ionic strength 0.5.

The above five extracts are denoted by the symbols I(KZ), II(KH), III(DMMA), IV(8T-3_2) and V(8T-DEAE), respectively.

All the extracts were passed down a Sephadex G-50 column to remove excess Triton X-100 before use in the permeability studies.

Permeability measurements. The unidirectional flux of radioactively-labelled monosaccharides across planar lipid bilayers formed from equimolar solutions of pure egg lecithin and cholesterol in *n*-decane was measured exactly as previously described [12,15]. For measurements made at 5°C the permeability cell was placed in a small water bath through which was circulated water from a thermostat.

Results

The composition of membrane extracts I(KZ) and their effect on glucose permeability. Fig. 1a shows an example of the elution profile of a Triton X-100 membrane extract prepared as described by Kahlenberg and Zala [9] and eluted from a DEAE column with eluant of ionic strength 0.1. The fractions under the peak were pooled, concentrated and re-chromatographed on a Sephadex G 50 column to give the elution profile shown in Fig. 1b. The object of this procedure was to remove excess Triton X-100. The fractions under the peak were again pooled, concentrated and used in permeability studies. The insets in Figs. 1a and 1b show the electrophoretograms obtained by SDS-polyacrylamide gel electrophoresis of the extracted ghost pellet and the final extract after chromatography on DEAE and G 50 columns. Kahlenberg and

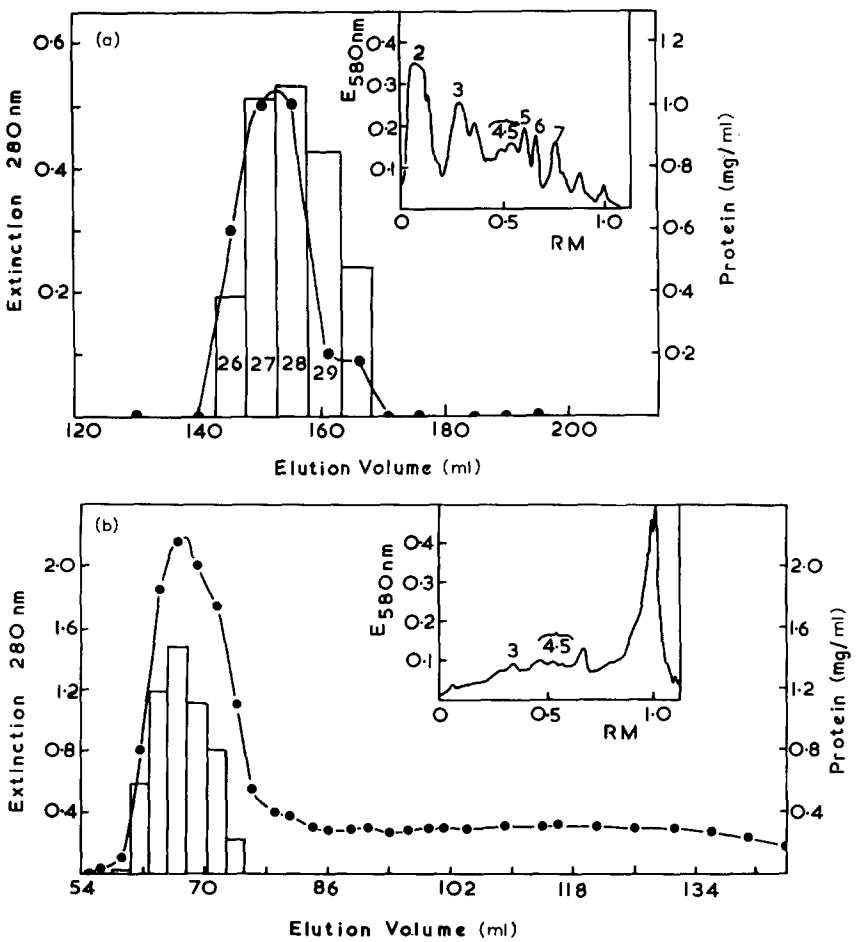

Fig. 1. a. Elution profile of human erythrocyte membrane extract produced by incubation of haemoglobin-free ghosts with 0.125% Triton X-100, *I* 0.02 and eluted from a DEAE-cellulose column with 0.1% Triton X-100, *I* 0.1 Inset: electrophoretogram of extracted ghost pellet. b. Elution profile of combined fractions 26—29 (a) on a Sephadex G 50 column. Inset: electrophoretogram of the pooled fractions. RM, relative mobility.

Zala [9] report that their procedure led to an extract containing protein predominantly in the 4.5 zone of the electrophoretogram, whereas in our hands we find a substantial amount of low molecular weight material; however, it must be borne in mind that we have introduced a further chromatographic step to remove detergent (see below). In agreement with Kahlenberg and Zala we find little band 3 polypeptide in this extract.

Table I shows permeability data for bilayer lipid membranes containing these extracts. For the particular extract of which the chromatography is illustrated in Fig. 1 (the second and third entries in Table I) there was no significant enhancement of D-glucose permeability but two further extracts prepared in the same fashion had transport activity. As in our previous work we regard enhancements of more than 1.9 to be significant. Kahlenberg and Zala [9] reported that the concentrated fraction eluted with medium of ionic strength 0.1 from the DEAE column was unstable and had to be rapidly reconstituted in liposomes to prevent loss of activity. They quote a decrease of activity of 48% in 2 h at 20°C. In our bilayer system the necessity to remove excess detergent and the time taken to carry out a permeability measurement means that experiments cannot be carried out rapidly. To test the stability of the extract permeability measurements were carried out on the day of extraction and on the following two days after extraction. The results in Table I show that the permeability measured on the day of extraction is more than twice as large as measured on the two successive days when the extract had apparently lost its transport activity.

TABLE I

D-GLUCOSE PERMEABILITIES OF BILAYER LIPID MEMBRANES AT 27°C

a, Standard deviation from 5 experiments; b and c, fraction obtained in experiments depicted in Figs. 1a and 1b; d, e and f, permeabilities of the same extract measured on the day of extraction and the two subsequent days respectively; g, h and i, permeabilities of the same extract measured on the day of extraction, one day later and 4 days later, respectively.

Membrane extract added	Protein concn. ($\mu g \cdot cm^{-3}$)	$P \times 10^8$ ($cm \cdot s^{-1}$)	P/P_0
None	—	3.86 ± 0.69(a)	1.00
I(KZ)	9.6	5.86(b)	1.5
I(KZ)	10.2	5.53(c)	1.4
I(KZ)	19.5	13.5	3.5
I(KZ)	13.8	7.52	2.0
I(KZ)	20.4	8.78(d)	2.3
I(KZ)	18.8	3.73(e)	1.0
I(KZ)	20.6	3.83(f)	1.0
II(KH)	40.4	40.5(g)	10.5
II(KH)	35.1	9.62(h)	2.5
II(KH)	8.2	3.11(i)	0.8
IV(8T-3_2)	35.4	7.38	1.9
IV(8T-3_2)	41.5	9.14	2.4
IV(8T-3_2)	58.4	8.44	2.2
V(8T-DEAE)	27.5	18.6	4.8
V(8T-DEAE)	40.8	17.1	4.4
V(8T-DEAE)	41.8	8.5	2.2

The composition of membrane extracts II(KH) and their effect on glucose permeability. Fig. 2a shows an example of the elution profile of a Triton X-100 membrane extract prepared as described by Kasahara and Hinkle [5,8] and eluted from a DEAE column with eluant of ionic strength 1.0. Fig. 2b shows the elution profile of the combined DEAE fractions when concentrated and re-chromatographed on a Sephadex G 50 column. Kasahara and Hinkle [5,8] found that an appreciable amount of active protein was washed off a DEAE column with 0.5% Triton X-100/50 mM Tris-HCl (their fraction I). We found that no significant amount of protein was eluted under the latter conditions. The insets in Figs. 2a and 2b show the electrophoretograms of the extracted ghost 'pellet' and the final extract after chromatography on DEAE and G 50 columns. The extract contains an appreciable amount of protein in what can probably be broadly defined as zone 4.5 and also some low moleulcar weight material giving a band similar to that found in extract I(KZ).

Table I shows that on the day of preparation extract II(KH) significantly

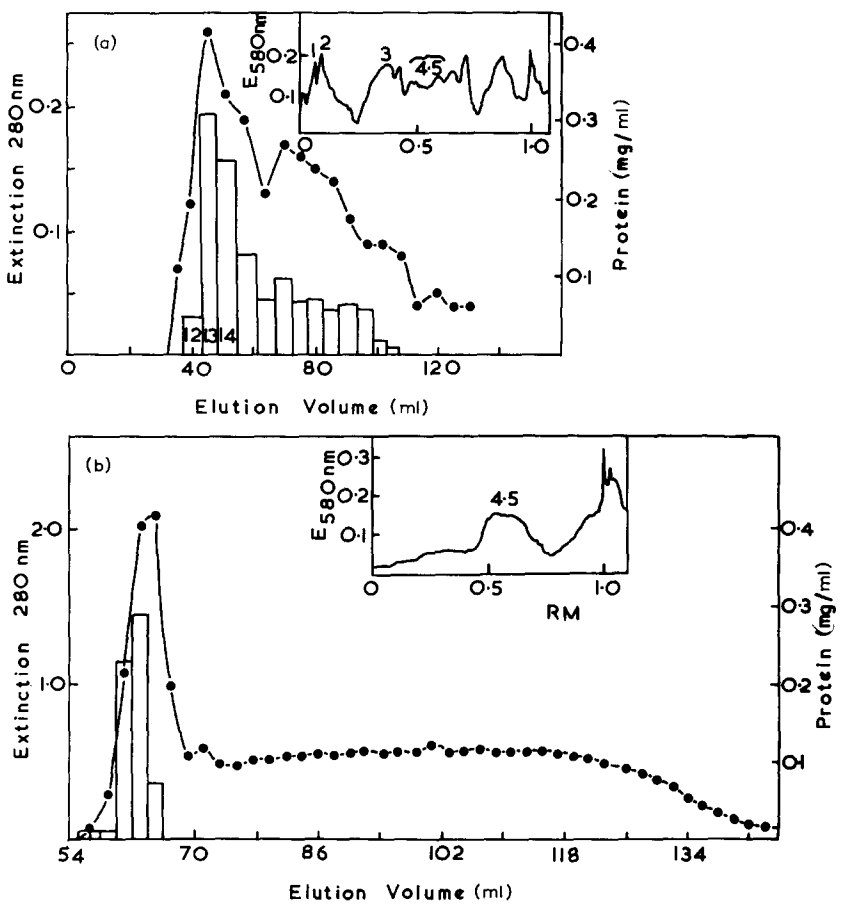

Fig. 2. a. Elution profile of human eryrhrocyte membrane extract produced by incubation of haemoglobin-free spectrin-depleted ghosts with 0.5% Triton X-100 and eluted from a DEAE-cellulose column with 0.5% Triton X-100, *I* 1.0. Inset: electrophoretogram of extracted ghost pellet. b. Elution profile of combined fractions 12—14 (a) on a Sephadex G 50 column. Inset: electrophoretogram of the pooled fractions. RM, relative mobility.

increased the permeability of bilayer lipid membrane to D-glucose but after storage for 24 h at 4°C the transport activity fell to approximately 24% of its initial value. A similar extract had no transport activity after storage for 4 days.

The composition of membrane extracts IV(8T-3_2) and their effect on glucose permeability. Fig. 3 shows the effect of the oxidizing agent, *o*-phenanthroline-cupric ion complex, on the electrophoretograms of spectrin-depleted ghosts. As seen in Fig. 3b the oxidizing agent results in a decrease in the amount of stainable polypeptides in the region of band 3 and the appearance of an intense band of higher molecular weight which we attribute to the crosslinked dimeric band 3 material [3]. Treatment of the ghosts with mercaptoethanol removes the band 3 dimer (Fig. 3c). Fig. 4 shows an analogous series of electrophoretograms for the spectrin-depleted ghost pellet and supernatant extract after incubation with 0.5% (w/v) Triton X-100 in 8 mM Tris-HCl buffer (i.e. an '8T extract' [15]). The supernatant was found to contain an appreciable proportion of material in the 4.5 region.

Table I gives the results of permeability experiments using 8T extracts of cross-linked ghosts. The measurements, which were made on the day of extraction, indicate that these extracts retain little transport-active material.

The composition of membrane extracts V(8T-DEAE) and their effect on glucose permeability. Fig. 5 shows the elution profile given by an 8T extract on chromatography with a DEAE-cellulose column. Some material came off of the column with the washing buffer but the major proportion was eluted with buffer of ionic strength 0.5. The largest fraction (no. 24) was re-chromatographed on Sephadex G 50 to remove excess detergent and used for permeability measurements. The electrophoretogram of fraction 24 shows that it contained a large proportion of polypeptide in zone 4.5. The increases in permeability produced on incorporation of the extract into lipid bilayers were significant (Table I).

The composition of membrane extracts III(DMMA) and their effect on glucose permeability. Table II shows permeabilities of bilayer lipid membranes with incorporated 8T extracts of dimethyl maleic anhydride-treated spectrin-depleted ghosts. At 27°C significant enhancement of D-glucose permeability was found with freshly prepared extracts. Because of the apparent lability of the transport system some measurements were made at 5°C. The enhancement of permeability was high at this temperature. The results with L-glucose indicate that, as previously found [15], the process is stereospecific. The effects

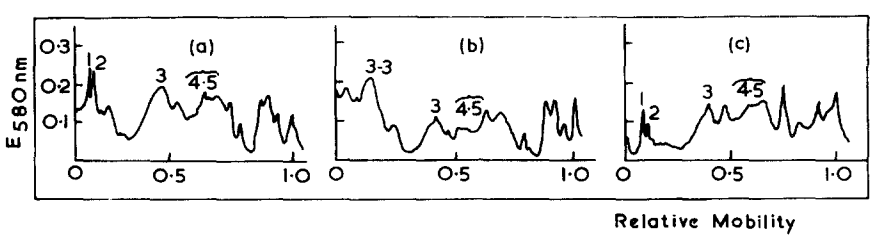

Fig. 3. SDS-polyacrylamide gel electrophoresis of human erythrocyte spectrin-depleted ghosts. a, control, 7.5 mM phosphate, pH 7.5; b, 7.5 mM phosphate, pH 7.5, 100 μM *o*-phenanthroline, 20 μM $CuSO_4$; c, as b plus 100 mM mercaptoethanol.

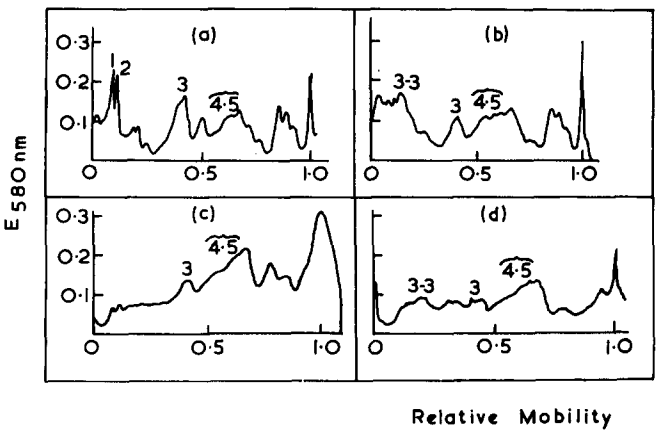

Fig. 4. SDS-polyacrylamide gel electrophoresis of Triton X-100 (0.5% (w/v) in 8 mM Tris-HCl) extracts of human erythrocyte spectrin-depleted ghosts. a, control pellet after extraction; b, cross-linked pellet; c, control supernatant; d, supernatant after treatment with cross-linking agent (100 μM *o*-phenanthroline, 20 μM $CuSO_4$).

observed are due to protein incorporation into the bilayer, the D-glucose permeability of the bilayer in the presence of Triton X-100 at 5°C is not changed significantly at free detergent concentrations of approx. 6 $\mu g \cdot ml^{-1}$, which is in excess of free Triton present in the membrane extracts.

Fig. 6 shows the elution profile of a III(DMMA) extract eluted from a Sephadex G 50 column; separation of the free Triton is evident. Of the three

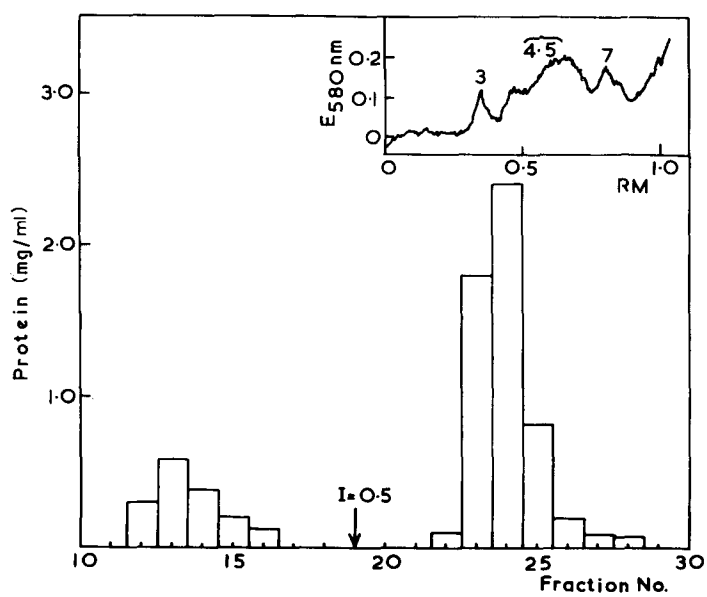

Fig. 5. Elution profile of human erythrocyte membrane extract produced by incubation of haemoglobin-free ghosts with 0.5% (w/v) Triton X-100 in 8 mM Tris-HCl, pH 8.0, and eluted from a DEAE-cellulose column. The arrow indicates the change of elutant to 0.5% (w/v) Triton X-100 in 8 mM Tris-HCl, *I* 0.5. Inset: electrophoretogram of fraction No. 24. RM, relative mobility.

TABLE II

GLUCOSE PERMEABILITIES OF BILAYER LIPID MEMBRANES INCORPORATING TRITON EXTRACTS OF DIMETHYL MALEIC ANHYDRIDE-TREATED ERYTHROCYTE MEMBRANES

Protein concn. ($\mu g \cdot cm^{-3}$)	Permeant	Temperature (°C)	$P \times 10^8$ ($cm \cdot s^{-1}$)	P/P_0
9.1	D-glucose	27	33.6	8.7
10.7	D-glucose	27	15.0	3.9
21.2	D-glucose	27	18.6	4.8
25.2	D-glucose	27	13.0	3.4
—	D-glucose	5	0.67 ± 0.05	1.00
4.98	D-glucose	5	6.73	10.0
4.88	D-glucose	5	4.17	6.2
13.3	D-glucose	5	16.1	24.0
25.8	D-glucose	5	4.39	6.5
—	L-glucose	5	1.1 ± 0.4	1.00
21.9	L-glucose	5	2.03	1.8

fractions containing most of the protein the first one (A) was used in a permeability experiment and gave an enhancement of 6.2 (see Table II). Inset in Fig. 6 is the electrophoretogram of the major protein-containing fraction (B). This fraction gave a major band in the 4.5 zone as well as an appreciable amount of lower molecular weight material. Quantities required for the permeability and electrophoresis did not allow us to carry out both experiments on the same fraction. However, in a repeat experiment, which gave an almost identical elution profile to that shown in Fig. 6, fraction B was used for a permeability experiment, the results of which are shown in Fig. 7 in comparison with results for a bilayer in the absence of protein extract. This gave a permeability enhancement of 24.0 (Table II).

Electrophoretograms of the type illustrated in Fig. 6 when compared with those obtained immediately after preparation of the extracts led us to investi-

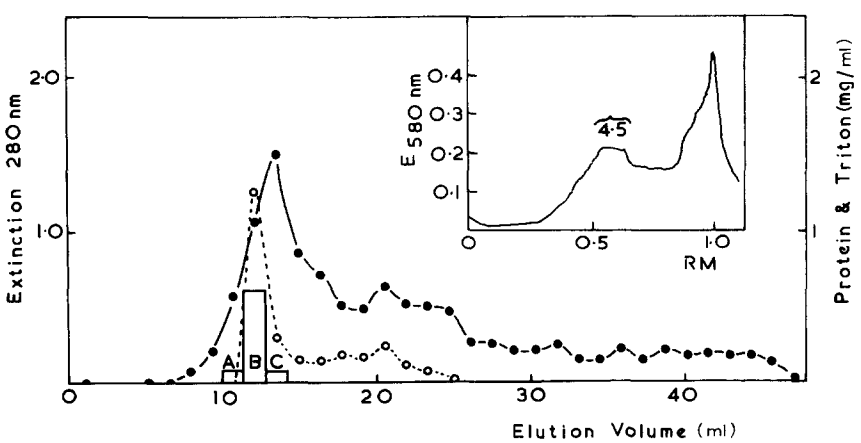

Fig. 6. Elution profile of Triton X-100 extract of dimethyl maleic anhydride-treated human erythrocyte haemoglobin-free spectrin-depleted ghosts eluted from a Sephadex G-50 column. Solid line and blocks, extinction at 280 nm and protein concentration (Lowry); dashed line, Triton X-100 concentration. Inset: electrophoretogram of fraction B. RM, relative mobility.

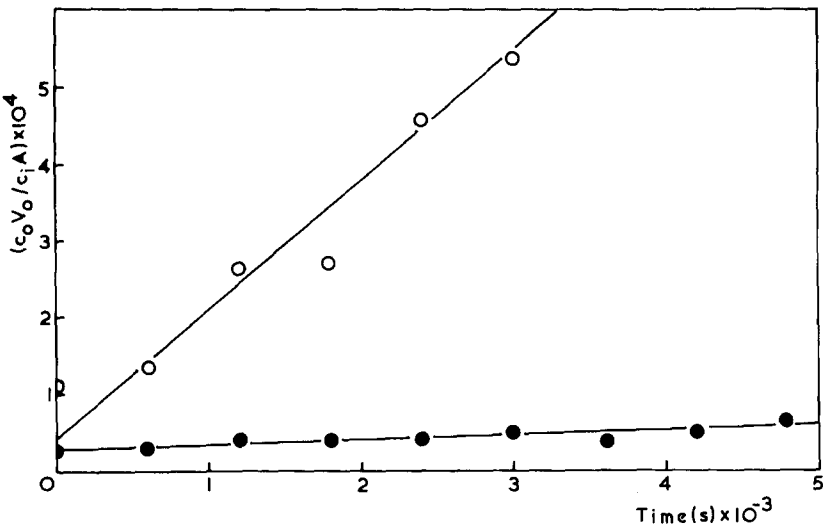

Fig. 7. Permeability plots at 5°C for bilayer lipid membranes. $V_o c_o$ is the amount of labelled sugar (total concentration c_i) passing through a bilayer of area A in time t. •, Passive transport of D-glucose; ○, facilitated transport of D-glucose on addition of Triton X-100 extract of dimethyl maleic anhydride-treated ghosts to the *trans* side of the bilayer. Protein concentration, 13.3 $\mu g \cdot cm^{-3}$.

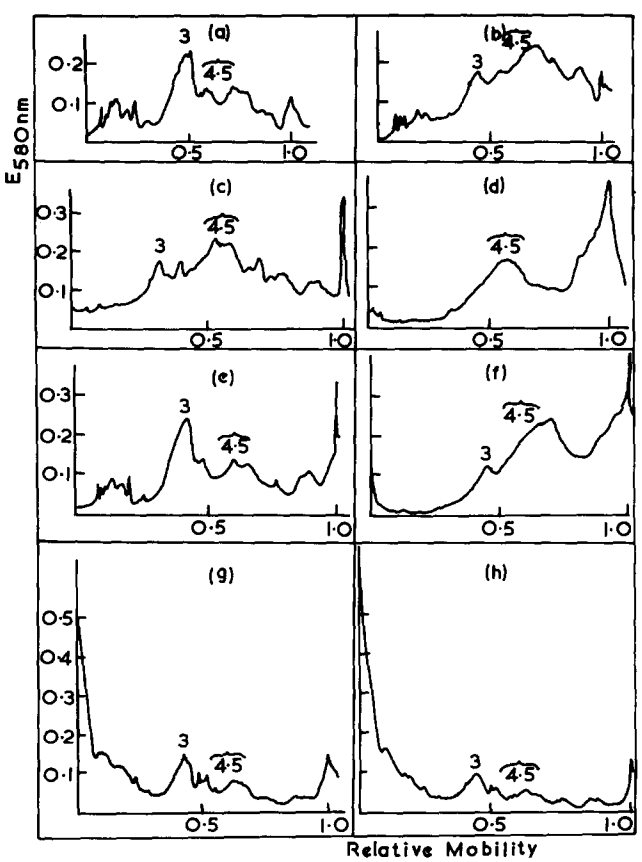

Fig. 8. SDS-polyacrylamide gel electrophoresis of Triton X-100 extracts of dimethyl maleic anhydride-treated human erythrocyte haemoglobin-free spectrin-depleted ghosts. a, c and e, gels run on the day of extraction; b and d, gel pattern after elution from a G 50 Sephadex column; f, gel pattern of extract after storage for 16 days at 4°C; g, gel pattern of freshly prepared extract after incubation of extract at 60°C for 15 min; h, as for g, incubation time 60 min.

gate the stability of the extracts in more detail. The results of some of these experiments are shown in Fig. 8 which shows results obtained from three independent membrane extractions. On the day of extraction the electrophoretograms of the extracts generally showed a strong band 3 with smaller amounts of material in the region of zone 4.5 (Fig. 8, a, c and e). However, the electrophoretograms of the major protein-containing fractions eluted from a Sephadex G 50 column contained a large amount of material in zone 4.5 (Fig. 8, b, d, and f) and often material of lower molecular weight (Fig. 8, b and d). Storage of the extract at 4°C for an extended period (16 days) results in an electrophoretogram (Fig. 8f) which had a very similar profile to that obtained after G 50 chromatography of a freshly prepared extract. Heating a freshly prepared extract at 60°C for between 15 and 60 min resulted in aggregation and electrophoretograms with a large proportion of high molecular weight material (Fig. 8, g and h). It is clear from these experiments that the III(DMMA) extract can undergo some type of disproportionation/degradation on storage at 4°C, and that the formation of low molecular weight material is facilitated by removal of excess Triton on G 50 chromatography. Addition of sodium azide to the extracts (0.05%) did not inhibit this disproportionation but storage at −20°C or addition of phenylmethylsulphonyl fluoride in the presence of calcium ions [21] resulted in electrophoretograms showing the absence of low molecular weight material which resembled thoses in Fig. 8g and h even after storage at 4°C.

Discussion

Of the two procedures reported to produce purified zone 4.5 polypeptides [8,9] in our system that of Kasahara and Hinkle [9] produced the more convincing results. The procedures differ in two respects; for extract I(KZ) the starting material is haemoglobin-free ghosts and the ion-exchange column is eluted with mediun of ionic strength 0.1, whereas for extract II(KH) the starting material is depleted of spectrin and the ion-exchange column eluted with medium of ionic strength 1.0. In agreement with Kahlenberg and Zala [8] we find no well defined band 3 polypeptides in extracts prepared using their procedure. The results in Table I imply a rapid loss of transport activity with time, in both types of extract. A direct comparison between the electrophoretograms of the extracts shown in Figs. 1b and 2b and the permeability enhancements observed with fresh extracts leads to the conclusion that transport activity is very likely associated with the broad 4.5 band polypeptides rather than the lower molecular weight material which is present in both extracts. Kasahara and Hinkle [9] reported an electrophoretogram showing zone 4.5, a small amount of band 7 and a large low molecular weight band which they attributed to lipid.

Comparison of the electrophoretograms depicted in Fig. 4 shows that cross-linking, while reducing the amount of band 3 in favour of the dimer in both the extracted membrane (pellet) and the supernatant, appreciably decreases the amount of low molecular weight material, implying that perhaps some of the low molecular weight materials arises from band 3 polypeptides. The permeability enhancements produced by the cross-linked extract are very small and

on average are only just above the level of significance.

In our previous work [15] we found that the most dramatic enhancements of D-glucose permeability occurred when extracts prepared from whole ghosts by incubation in 8 mM Tris-HCl buffer (i.e. 8T extracts) were incorporated into bilayers. Ion-exchange chromatography of 8T extracts, an example of which is illustrated in Fig. 5, leads to material giving electrophoretograms showing a small band 3, a broad zone 4.5 and band 7. These extracts generally give very significant enhancements in bilayer permeability (Table I).

Lin and Spudich [2] reported that dimethyl maleic anhydride solubilized all the major membrane proteins of the human erythrocyte excepts bands 3 and 7. Using their procedure followed by a Triton X-100 extraction in 8 mM Tris-HCl buffer we generally found a large proportion of band 3 but also other bands, notably some zone 4.5. Chromatography of these extracts on Sephadex G 50 results in a predominance of zone 4.5 and low molecular weight material. These extracts gave large enhancements of D-glucose permeability at both 27 and 5°C.

A comparison of the electrophoretograms shown in Figs. 2b, 5, 6, 8b and d of extracts eluted from a G 50 column (or a DEAE-cellulose column; Fig. 5) shows that they all give a broad zone 4.5, sometimes residual amounts of band 3 (Figs. 5, 9b) and varying amounts of low molecular weight material in the region of the tracking dye. A similar pattern is found after storage of a DMMA extract for several days at 4°C without G 50 chromatography. The results shown in Fig. 8 for fresh extracts before and after G 50 chromatography indicate that much of the material defined as zone 4.5 and the low molecular weight band arises from higher molecular weight polypeptides including the breakdown of band 3. In this connection it should be noted that higher resolution polyacrylamide gel electrophoresis using a discontinuous buffer system reveals approx. 7 individual bands in zone 4.5 [22,23]. The mechanisms involved in this breakdown cannot be unambiguously established from the present studies. Since the process is accelerated by removal of excess Triton X-X-100 and is unaffected by addition of sodium azide but is inhibited at −20°C and, to some degree, by addition of phenylmethylsulphonyl fluoride it is likely that proteolysis possibly involving one or more enzymes may be involved. At 60°C aggregation is the dominant process rather than proteolysis.

Finally we consider the implications of these observations with respect to the identification of the polypeptide components responsible for facilitated diffusion of monosaccharides across the erythrocyte membrane. It should be emphasised that our approach to the identification problem is based on the assumption that the extracts we have prepared have comparable affinity for the bilayer; although we have shown that extracts affect the electrical properties of the bilayer [15] we have at present no direct information on the extent of incorporation of a given extract into the bilayer. On the basis of electrophoretograms of extracts prior to removal of excess Triton X-100 we previously concluced [13—15] in agreement with others [2—7] that band 3 polypeptides were most likely to be involved in monosaccharide transport. The present observations lead us to qualify this view. It is clear that transport activity can arise when little or no band 3 polypeptides are detectable in the extracts after G 50 chromatography; however our experiments show that it is very likely that some proportion of the material detected in zone 4.5 is derived

from the breakdown of band 3 polypeptides. Two extreme possibilities could lead to the artifactual involvement of band 3 breakdown products. Firstly, if the proteolysis of band 3 produced only low molecular weight material, and secondly, if transport activity was related to some other proteolysis products present in the extracts. However, the formed possibility seems unlikely and since the reconstituted transport system prossesses many of the properties of the in vivo system including stereospecificity the latter possibility can be eliminated. Furthermore, studies devoted to the characterization of band 3 have shown that proteolytic fragments of molecular weights in the range of approx. 40 000—55 000 which would be expected to migrate to zone 4.5 are produced by chymotrypsin and trypsin [24,25]. Pronase digestion of the erythrocyte membrane results in cleavage of band 3 polypeptides at sites external to the membrane inteface leading to an intense band in zone 4.5 with a molecular weight of approx. 60 000 identified with the transmembrane fragments of band 3 [24]. The latter could be associated with transport function. If the components of the monosaccharide transport system were initially present in band 3 polypeptides and retained some of their function after proteolysis to the lower molecular weight species present in the enhanced zone 4.5 then all the earlier evidence [2—7] implicating band 3 polypeptides with the monosaccharide transport system would be reconcilable with the results reported here and with the reconstitution experiments with liposomes [8,9].

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