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ELECTRICAL PROPERTIES AND GLUCOSE PERMEABILITY OF BILAYER LIPID MEMBRANES ON INCORPORATION OF ERYTHROCYTE MEMBRANE EXTRACTS

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Summary

1. Extracts of the human erythrocyte membrane have been prepared by solubilization with Triton X-100 and analysed by electrophoresis and gel filtration techniques.

2. The extracts have been incorporated asymmetrically into lecithin-cholesterol-*n*-decane planar bilayers.

3. The electrical characteristics and glucose permeabilities of the bilayers have been measured.

4. The extracts increased the electrical conductance of the bilayers and also markedly enhanced the D-glucose permeability but not the L-glucose permeability.

5. The enhanced D-glucose permeability was inhibited by monosaccharide transport inhibitors.

6. The results support the claim that a monosachharide facilitated diffusion system has been set-up in vitro which has many of the characteristics of the transport system in the human erythrocyte membrane.

7. The data indicates that the trans membrane polypeptides of band 3 of the electrophoretogram of the erythrocyte membrane proteins (notation of Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2616) are implicated in D-glucose transport, although the possibility that a relatively minor component of the membrane could be responsible for glucose transport cannot be eliminated.

Introduction

There have been several studies of the effects of human erythrocyte membrane protein extracts on the electrical properties of planar bilayer lipid

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membranes. Cherry et al. [1] and van der Berg [2] demonstrated increased conductance on incorporation of the major apoproteins extracted using butanol-water partition. Increased bilayer lipid membrane conductances were also observed by Lossen et al. in the presence of both a 'strongly bound' fraction [3] (consisting of protein molecular weight 95 000 and the sialoglycoproteins) and a 'loosely bound' fraction [4]. Incorporation of the major sialoglycoprotein of human erythrocytes into bilayer lipid membranes made from sheep erythrocyte lipids was found to lead to an increased conductance which was further enhanced by the addition of concanavalin A [5].

The relatively diverse nature of the protein extracts giving rise to enhanced conductance (ion transport) would seem to imply that this is to some extent a general phenomenon associated with protein incorporation in bilayers. In contrast the transport of non-electrolytes such as monosaccharides across the human erythrocyte membrane is believed to involve a specific carrier protein or proteins constituting a facilitated diffusion system. There have been unsuccessful attempts to reconstitute this system in planar bilayers [6,7]. Bilayer lipid membranes have a very low permeability to sugars ($\approx 10^{-8}$ cm $\cdot$ s^{-1}) [6—8] so that in principle they offer a very sensitive means of detecting the transport activity of incorporated proteins and hence of identifying the carrier species.

There are several reports on the incorporation of erythrocyte membrane proteins into liposomes [9—11] giving rise to preferential uptake of D-glucose characteristic of the erythrocyte monosaccharide transport system. The published results largely support the view that a constituent or constituents of band 3 (nomenclature of Fairbanks et al. [12]) seen in the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis pattern of erythrocyte ghosts is responsible for monosaccharide transport activity. Thus the association of band 3 polypeptides with glucose transport activity is supported by binding experiments reported by Kahlenberg and Walker [13], Lin and Spudich [14] and Taverna and Langdon [15] although Masiak and Le Fevre [16] using proteolytic enzymes to digest the erythrocyte membrane inner surface have implicated the cytoplasmic membrane protein 'spectrin': (bands 1 and 2) with glucose transport. However, this result does not agree with the conclusions reached by Carter et al. [17] on the basis of proteolysis experiments nor with Zoccoli and Lienhard [18] who studied protein-depeleted inside-out vesicles produced by alkali treatment which removed spectrin but did not affect sorbose transport. This conflict has been complicated by the observation that the lower molecular weight proteins of band 4.5 are associated with glucose transport (ref. 19 and C.A. Zala, personal communication). It should also be noted that band 3 proteins have been associated with anion permeability [20—23], ATPase activity [24,25] and cytochalasin B binding [14]. These studies clearly demonstrate the heterogeneous nature of this group of trans membrane proteins.

There appears to have been only a single report on the reconstitution of a sugar transport system in bilayer lipid membranes. Storelli et al. [26] incorporated the intestinal sucrase-isomaltase complex into bilayers and demonstrated enhanced permeability to glucose and fructose arising from sucrose. We report here the electrical characteristics and D-glucose permeabilities of lecithin-cholesterol-*n*-decane bilayers containing Triton X-

100-solubilized extracts of human erythrocyte membranes. We have produced bilayers with D-glucose permeabilities enhanced by two orders of magnitude. A preliminary report of some of the permeability measurements has been published [27].

Experimental

Preparation of membrane extracts. All the membrane extracts were prepared from haemoglobin-free 'ghosts' obtained by a modification of the method of Dodge et al. [28] and were analysed by SDS-polyacrylamide gel electrophoresis according to the procedure of Fairbanks et al. [12]. The gels were scanned at 550 nm on a Unicam SP500 spectrophotometer. Protein contents of the extracts were measured by modified Lowry assays for detergent-containing extracts [29] and detergent-free extracts [30]. Phospholipid was assayed by the zinzade method [31] and Triton X-100 by the method of Garewal [32]. The following extracts were obtained: (1) A crude extract which we denote as the '8T extract' [33]. This was obtained by suspending the ghosts in a 8 mM Tris · HCl buffer, pH 8.0, to a protein concentration of 15 mg/cm^3 followed by incubation with a 5-fold volume of 0.5% (w/v) Triton X-100 in the same buffer for 20 min at 0°C. (2) A spectrin-free band 6-depleted extract which we denote as the '40T extract' [34]. This was obtained by successive incubation of ghosts with 0.1 mM EDTA for 30 min at 30°C, 0.25 mM Tris · HCl buffer, pH 8.0, for 1 h at 0°C and 0.1 M Tris · HCl buffer, pH 8.0, for 30 min at 0°C, followed by incubation with a 5-fold volume of 0.5% (w/v) Triton X-100 in 40 mM Tris · HCl buffer, pH 8.0, for 20 min at 0°C. (3) A further purified 40T extract obtained by ion-exchange chromatography using a Whatman DEAE-cellulose DE-52 column [34]. Excess Triton X-100 was removed from all the extracts on a Sephadex G-50 column.

Spectrin was prepared by concentration of 0.1 mM EDTA extracts of ghosts by ultrafiltration (Amicon PM 10 filter). Band 6 polypeptides were prepared by concentration of 0.25 M and 0.1 M Tris · HCl washings of ghosts. Both spectrin and band 6 polypeptides were dialysed against 8 mM Tris · HCl buffer, pH 8.0, for 24 h at 4°C. Lipid preparations from ghosts and the 40T extract were made by chloroform/methanol extraction.

Preparation of solutions for bilayer formation. Equimolar solutions of chromatographically pure egg lecithin and cholesterol in *n*-decane were prepared as previously described [7] and stored under nitrogen at −20°C.

Electrical measurements. The electrical capacitance and resistance of the bilayers were measured in the apparatus previously described [7]. Membrane resistances were measured by the d.c. method [35] using calomel electrodes (Lock and Co. Ltd. Type 11162) linked to the solutions bathing each side of the bilayer by 0.1 M KCl-agar bridges. Capacitance measurements were made either by the d.c. method or by the a.c. bridge method [33] using a Wayne-Kerr bridge (B224). The relative permittivity of the bilayer was taken as 2.4 [36].

Permeability measurements. The undirectional flux of radioactively labelled monosaccharides across the bilayer was measured during equilibrium exchange at sugar concentrations of 30 mM in balanced salt solutions as previously

described [7]. Briefly the apparatus consisted of two compartments separated by a teflon partition with an orifice to support the bilayer. The inner closed compartment was preloaded with labelled sugar solution, the membrane was formed, the solution in the outer open compartment was exchanged for unlabelled sugar solution and the rate of translocation of labelled sugar was monitored as a function of time by removing aliquots from the outer compartment. The calculation of the permeability coefficients (P) was based on the equation

$$P = \frac{V_0 c_0}{t A c_i} \tag{1}$$

where $V_0 c_0$ is the amount of labelled material which passes through the bilayer of area A in time t and c_i is the total concentration of labelled sugar in the system. The purity of the radiolabelled sugars, D-[U-^{14}C]glucose (Radiochemical Centre, Amersham, U.K.; specific activity 270—290 Ci/mol) and L-[1-^{14}C]-glucose (New England Nuclear, Boston, Mass.; specific activity 51.6 Ci/mol) were checked by paper chromatography using Whatman No. 1 paper. The paper, previously washed with diluted HCl followed by distilled water and dried at 80°C, was equilibrated with a butanol/ethanol/H_2O (4 : 1 : 5, v/v) mixture. The aqueous phase was 0.5 mM EDTA/0.2% (v/v) formic acid. Inorganic ions in the radiotracer solution were removed with a mixed bed ion-exchange resin (Amberlite 1R 4B + 1R 120). Approx. 1 μCi of the sugars in 20 μl of solution was applied to the paper and the chromatogram was developed for 4 h, dried and scanned on a Packard Model 7201 Radiochromatogram counter. The sugars were found to be at least 99% pure.

Two balanced salt solutions were used for the bathing solution; (A) 125 mM NaCl/5 mM KCl/3.8 mM $CaCl_2$/2.5 mM $MgCl_2$/5 mM NaN_3/5 mM Tris · HCl (pH 7.4) and (B) 140 mM NaCl/5 mM KCl/5 mM NaN_3/5 mM Tris · HCl (pH 7.4).

Gel filtration. Membrane extracts were studied using a Sepharose 6B column (2.5 cm internal diameter × 60 cm) with a bed volume of 280 ml and eluted with 0.1% Triton X-100/40 mM Tris · HCl (pH 8.0) at 4°C at a rate of 0.5 $cm^3 \cdot min^{-1}$.

Results

SDS-polyacrylamide gel electrophoresis of membrane extracts

Fig. 1 shows the electrophoretograms of haemoglobin-'free' ghosts and the membrane extracts. The peaks were assigned according to the notation of Fairbanks et al. [12]. An attempt was made to estimate the percentage of the major peaks from their areas and these results are given under the respective peaks. Fig. 1(b) shows that the 8T extract is a relatively crude preparation. It is deficient in bands 1, 2, 4.2, 5, 7 and 8 as compared with the haemoglobin-'free' ghost electrophoretogram (a). The 40T extract contains predominantly bands 3 and 4.2 and is substantially depleted in the other polypeptides. Purification of this extract using DEAE-cellulose chromatography gave a sample enriched in band 3 (d). Both the spectrin (bands 1 and 2) and band 6 polypeptide preparations gave the required products (e and f).

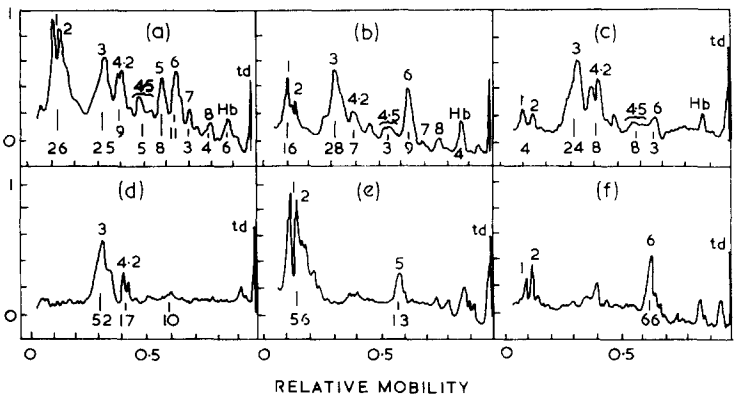

Fig. 1. Electrophoretograms of the polypeptides of the erythrocyte membrane and membrane extracts. The extinction of the Coomassie Brilliant Blue stain profile was measured at 550 nm; the peak assignments are given above the peaks (according to ref. 12) and the numbers below the peaks are the estimated percentages of each polypeptide band. (a) Haemoglobin-'free' ghosts, (b) 8T extract, (c) 40T extract, (d) DEAE-purified 40T extract, (e) spectrin polypeptides prepared by EDTA extraction, (f) band 6 polypeptides prepared from concentrated ghost washings. The amount of polypeptide added to each gel was in the range 20—95 μg.

The effect of 8T extracts on the thinning and thickness of bilayer lipid membranes

Fig. 2 shows the change in capacitance (a.c. method) of bilayers as a function of time in the absence and presence of 8T extracts on one side of the membrane. In these experiments zero time was taken when the membrane appeared uniformly silver when viewed in reflected white light. The 8T extract only slightly increased the rate of thinning but had no significant effect on the final equilibrium capacitance and hence thickness. In all cases black islands appeared in the membrane above a capacitance of approx. 0.05 $\mu F \cdot cm^{-2}$. Table I records the equilibrium capacitance and thickness of the bilayer, measurement of bilayer capacitance by the d.c. method gave slightly higher values of 0.474 ± 0.022 $\mu F \cdot cm^{-2}$ (20 determinations).

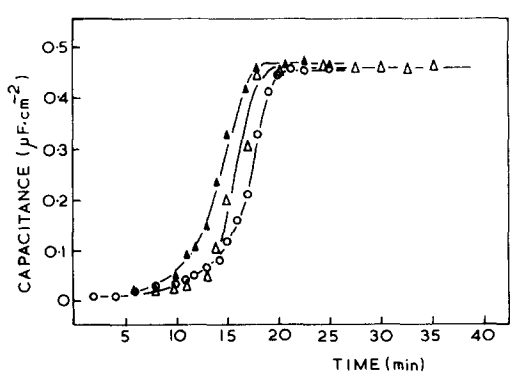

Fig. 2. Time dependence of membrane capacitance at 25°C, bathing solution 0.1 M NaCl, pH 7.4, ○, no protein extract; ▲, 8T extract added initially (protein concentration in bathing solution 25.2 $\mu g \cdot cm^{-3}$, Triton X-100 concentration 10.2 $\mu g \cdot cm^{-3}$); △, 8T extract added after thinning for 20 min (protein concentration 22.7 $\mu g \cdot cm^{-3}$, Triton X-100 6.7 $\mu g \cdot cm^{-3}$). The 8T extracts were added to one side of the membrane.

TABLE I

CAPACITANCE AND THICKNESS OF BILAYER LIPID MEMBRANES

Results are expressed as mean ± S.E.

Extract ($\mu g \cdot cm^{-3}$)	No. of experiments	Capacitance ($\mu F \cdot cm^{-2}$)	Thickness (nm)
None	7	0.449 ± 0.01	4.73 ± 0.20
8T extract (25.1 ± 2.4) *	3	0.449 ± 0.02	4.73 ± 0.31
8T extract (25.2 ± 10) **	3	0.456 ± 0.01	4.66 ± 0.23
Triton X-100 (1.0) **	2	0.450 ± 0.02	4.72 ± 0.31

* Added at zero time.
** Added at 5—10 min.

The effect of 8T extracts on the d.c. resistance of bilayer lipid membranes

Figs. 3a and 3b show typical plots of the decrease in bilayer resistance (d.c. method) on the addition of 8T extracts on one side of the membrane in bathing solutions containing K^+ and Na^+, respectively. The extracts were added to bilayers which had thinned to their equilibrium thickness. Since the extracts contained Triton X-100 it was important to establish what effect the detergent had on the electrical characteristics. Figs. 4a and 4b show the effect of 1 $\mu g/cm^3$ of 'free' detergent on one side of the bilayer, on the resistance and capacitance in the presence of K^+ and Na^+, respectively. Two features of these curves distinguish the effects of free detergent on bilayers from those of the 8T extracts. Firstly, the decrease in membrane resistance is transient and secondly the magnitude of the decrease is three orders of magnitude greater in the presence of K^+ than in the presence of Na^+. One the basis of this evidence it seems reasonable to conclude that the permanent increase in conductance observed for bilayers in the presence of 8T extracts arises from penetration of the protein · detergent complex into the bilayer and not from the very low concentration of free detergent which must be in equilibrium with it.

Figs. 5a and 5b show typical plots of the effect of putting 8T extracts, on both sides of bilayer in the presence of K^+ and Na^+, respectively. In general

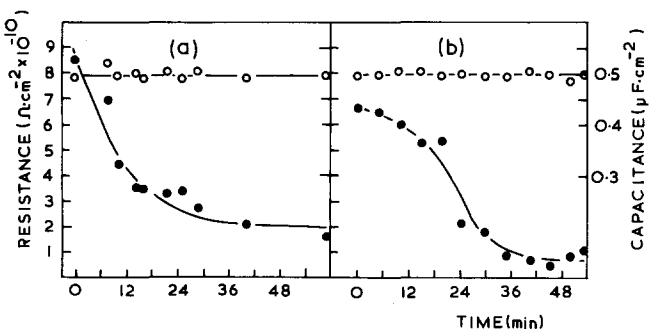

Fig. 3. Time dependence of resistance (●) and capacitance (○) of bilayers lipid membranes on asymmetric addition of 8T extracts at 27°C. (a) Bathing solution 0.1 M KCl, pH 6.4. Protein concentration 18 $\mu g \cdot cm^{-3}$, Triton X-100 concentration 12.5 $\mu g \cdot cm^{-3}$. (b) Bathing solution 0.1 M NaCl, pH 6.4. Protein concentration 17.2 $\mu g \cdot cm^{-3}$, Triton X-100 concentration 10.9 $\mu g \cdot cm^{-3}$.

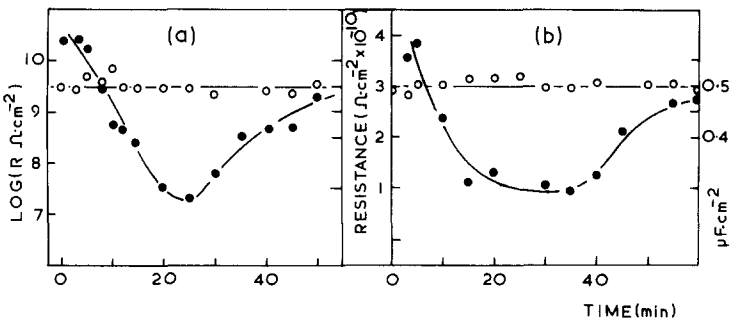

Fig. 4. Time dependence of resistance (•) and capacitance (○) of bilayer lipid membranes on asymmetric addition of Triton X-100 at 27°C. (a) Bathing solution 0.1 M KCl, pH 6.4 + 1.0 $\mu g \cdot cm^{-3}$ Triton X-100. (b) Bathing solution 0.1 M NaCl, pH 6.4 + 0.1 $\mu g \cdot cm^{-3}$ Triton X-100.

this resulted in a rapid decrease of approximately an order of magnitude in resistance followed by membrane rupture. Furthermore we found it practically impossible to form a membrane at all in bathing media containing the membrane extract.

In no case was there a significant effect of the membrane extract on bilayer thickness.

The effect of erythrocyte membrane extracts on glucose permeability

Figs. 6a and 6b show some represtative permeability plots for bilayers based on Eqn. 1. The slopes of the lines are equal to the permeability coefficients (P) or in the absence of extracts the passive permeability (P_0). The absolute positions of the lines on the graphs are not significant since they reflect the extent to which the level of activity in the outer compartment of the apparatus was reduced before measurements were begun. The permeability coefficients are recorded in Table II together with the ratio (P/P_0) which reflects the degree of enhancement of sugar permeability by the various membrane extracts. The average percentage standard error calculated by least squares from the

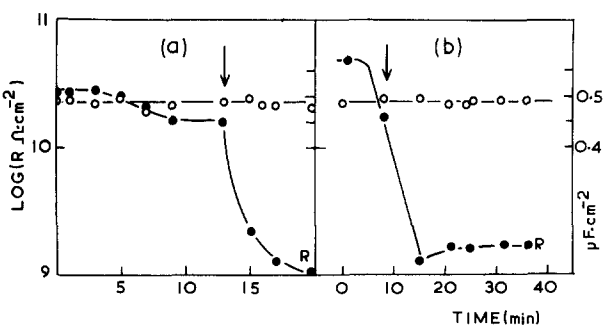

Fig. 5. Time dependence of resistance (•) and capacitance (○) of bilayer lipid membranes on symmetric addition of 8T extracts at 27°C. (a) Bathing solution 0.1 M KCl, pH 6.4. Protein concentration 37.5 $\mu g \cdot cm^{-3}$, Triton X-100 concentration 18.0 $\mu g \cdot cm^{-3}$. (b) Bathing solution 0.1 M NaCl, pH 6.4. Protein concentration 22.0 $\mu g \cdot cm^{-3}$, Triton X-100 concentration 12.0 $\mu g \cdot cm^{-3}$. The arrows indicate the time of addition of the 8T extracts to the trans side of a bilayer equilibrated with a 8T extract on the cis side. The electrical characteristics were measured until the membrane ruptured (R).

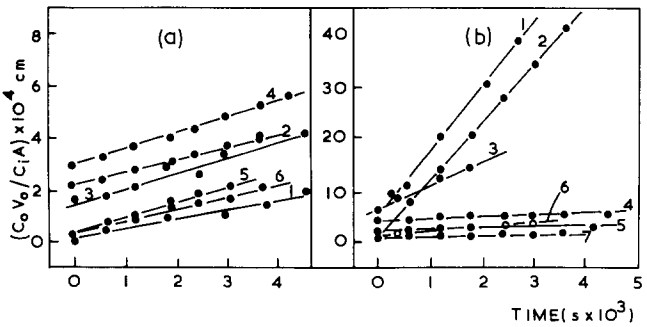

Fig. 6. Permeability plots for bilayer lipid membranes. V_0c_0 is the amount of labelled sugar (total concentration c_i) passing through a bilayer of area A in time t. (a) Curve 1, passive transport of D-glucose; 2, passive transport of L-glucose; curves 3—6 are for D-glucose transport in the presence of the following additives; 3, spectrin (10.3 $\mu g \cdot cm^{-3}$); 4, band 6 polypeptides (20.0 $\mu g \cdot cm^{-3}$); 5, Triton X-100 (1 $\mu g \cdot cm^{-3}$); 6, total erythrocyte membrane lipids (10.0 $\mu g \cdot cm^{-3}$). (b) D-glucose transport in the presence of the following additives; 1, 40T extract (7.9 $\mu g \cdot cm^{-3}$); 2, 40T extract (24.0 $\mu g \cdot cm^{-3}$); 3, DEAE-purified 40T extract (18.3 $\mu g \cdot cm^{-3}$); 4, 40T extract (14.9 $\mu g \cdot cm^{-3}$) + $HgCl_2$ (2 mM); 5, 40T extract (20.4 $\mu g \cdot cm^{-3}$) + *p*-chloromercuribenzoate (0.1 mM); 6, 40T extract (20.3 $\mu g \cdot cm^{-3}$) + phloretin (0.3 mM), 7, 40T extract (27.0 $\mu g \cdot cm^{-3}$) + 2-deoxy-D-glucose (120 mM). The figures after the extracts refer to the protein concentration. In all cases the membrane extracts were added to the trans side of the membrane.

permeability plots (excluding the data for the passive diffusion of D- and L-glucose) was 2.7%. The standard error calculated from repeated measurements of the passive diffusion of D-glucose was 8.3%. From these figures we estimate that the standard error in the ratio (P/P_0) is on the order of 0.8 so that values of $P/P_0 < 1.8$ should not be regarded as significant.

From these results the following points emerge; (1) the 8T, 40T and DEAE-purified 40T extracts significantly enhance the D-glucose permeability of the bilayers; (2) the sugar transport inhibitors *p*-chloromercuribenzoate, *p*-chloromercuribenzene sulphonate, mercuric chloride, and phloretin all inhibit D-glucose transport as does the competitive inhibitor 2-deoxy-D-glucose although inhibition is marginally less than might be expected in the latter case. (3) Materials which are associated with membrane extracts; spectrin, band 6 polypeptides (glyceraldehyde-3-phosphate dehydrogenase), ghost lipids and Triton X-100 do not enhance D-glucose permeability; (4) the L-glucose permeability of bilayers is not significantly enhanced by the 40T extract at concentrations which enhance D-glucose permeability.

Gel filtration chromatography of membrane extracts

Fig. 7 shows the elution profiles of the membrane extracts. Apart from the crude 8T extract the profiles of the other preparations consist essentially of an included peak of molecular weight ≈700 000 and an excluded peak molecular weight $>10^6$. The results indicate that the protein · detergent complex ($M_r \approx 700\,000$) undergoes an association which changes with both time and temperature and on addition of Ca^{2+}. Prolonged storage and elevated temperatures increases the proportion of excluded material as does incubation with Ca^{2+}. There appears to be some disproportionation and/or degradation after prolonged storage (Fig. 7e), at high temperature (Fig. 7f) and on incubation with Ca^{2+} (Fig. 7g) resulting in a peak with a molecular weight ≈30 000. The

TABLE II

MONOSACCHARIDE PERMEABILITIES OF BILAYER MEMBRANES

PCMB, *P*-chloromercuribenzoate; PCMBS, *P*-chloromercuribenzene sulphonate. The concentrations of extract given are those in the bathing solution on the trans side of the bilayer lipid membrane. The figures in brackets are the Triton X-100 concentrations in the extracts.

Permeant	Membrane extract	Concn. ($\mu g \cdot cm^{-3}$)	Additive	$P \times 10^8$ ($cm \cdot s^{-1}$)	P/P_O
D-Glucose	—	—	—	4.51 ± 0.95 *,**	1.00
L-Glucose	—	—	—	8.65 ± 0.95 **	1.00
D-Glucose	8T	5.1	—	32.7 *	7.3
D-Glucose	8T	11.0	—	13.9 *	3.1
D-Glucose	8T	11.0	—	28.7 *	6.4
D-Glucose	8T	53.2 (37.2)	—	203.2 *	45.1
D-Glucose	8T	90.1 (50.3)	—	212.1 *	47.1
D-Glucose	40T	5.1 (≤1)	—	114.8	25.5
D-Glucose	40T	6.0 (≤1)	—	35.2	7.8
D-Glucose	40T	7.2 (5.4)	—	45.8	10.2
D-Glucose	40T	7.9 (≤5)	—	129.1	28.6
D-Glucose	40T	10.4 (7)	—	66.9	14.8
D-Glucose	40T	17.9 (16.1)	—	135.0 *	29.9
D-Glucose	40T	24.0	—	114.5 *	25.4
D-Glucose	DEAE-40T	10.0 (7.1)	—	50.4 *	11.2
D-Glucose	DEAE-40T	12.1 (≤5)	—	16.1 *	3.6
D-Glucose	DEAE-50T	38.5	—	38.5 *	8.6
D-Glucose	40T	20.2 (15.0)	PCMBS (1 mM)	4.84	1.1
D-Glucose	40T	23.1 (17.3)	PCMBS (1 mM)	5.25	1.2
D-Glucose	40T	10.1	PCMB (0.1 mM)	4.21	0.9
D-Glucose	40T	20.4	PCMB (0.1 mM)	4.23	0.9
D-Glucose	40T	14.9 (10)	$HgCl_2$ (2 mM)	5.23	1.2
D-Glucose	40T	10.1 (5)	$HgCl_2$ (2 mM)	4.28	1.0
D-Glucose	40T	20.3	phloretin (0.3 mM)	4.06	0.9
D-Glucose	40T	18.7	phloretin (0.47 mM)	6.99	1.6
D-Glucose	40T	27.0	2-deoxy-D-glucose (120 mM)	8.08	1.8
D-Glucose	40T	21.0	2-deoxy-D-glucose (120 mM)	9.62	2.1
L-Glucose	40T	18.2	—	9.06	1.0
L-Glucose	40T	27.7	—	15.19	1.8
D-Glucose	Spectrin	10.3	—	4.50	1.0
D-Glucose	Spectrin	12.3	—	4.97	1.1
D-Glucose	Band 6	20.1	—	5.77	1.3
D-Glucose	Band 6	40.2	—	4.95	1.1
D-Glucose	Ghost lipids	10.8	—	4.77	1.1
D-Glucose	Ghost lipids	10.3	—	4.44	1.0
D-Glucose	40T extract lipids	10.0	—	4.73	1.1
D-Glucose	Triton X-100	1.14	—	7.79	1.7
D-Glucose	Triton X-100	1.04	—	7.11	1.6

* Bathing solution contained divalent ions.

** Standard deviation based on multiple measurements.

major components of the 40T extract are bands 3 and 4.2 which have molecular weights according to SDS-polyacrylamide gel electrophoresis of ≈90 000 and ≈40 000, respectively. Band 3 is believed to be dimeric in the erythrocyte membrane and associated with the cytoplasmic tetrameric protein which gives rise to band 4.2 [33]. On this evidence a complex consisting of two band 3 polypeptides (M_r = 180 000) and four band 4.2 polypeptides (M_r = 288 000) requires an amount of Triton X-100 corresponding to a molecular weight ≈240 000, to give a total molecular weight of ≈700 000. The complex would

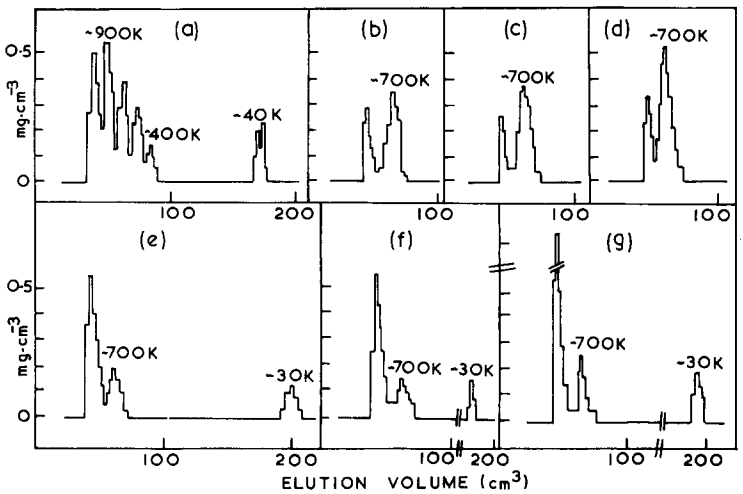

Fig. 7. Gel filtration chromatography on a Sepharose 4B column of human erythrocyte membranes. (a) 8T extract. (b) DEAE-purified 40T extract. (c) 40T extract after storage in liquid nitrogen immediately after preparation. (d) 40T extract after 48 h at 4°C. (e) 40T extract after 7 days at 4°C. (f) 40T extract after heating for 10 min at 80°C. (g) 40T extract after incubation at 25°C for 20 min with 3.8 mM Ca^{2+}. In each case the column was eluted with 0.1% Triton X-100/40 mM Tris · HCl, pH 8.0, buffer.

then contain ≈34% by weight of Triton X-100. This figure is in the range reported by Clarke [37] for protein · Triton complexes.

Discussion

The permeability results support the claim that a glucose transport system having characteristics of the facilitated diffusion system of the human erythrocyte membrane has been set-up in vitro. The process is stereospecific for D-glucose and is inhibited by D-glucose transport inhibitors. The permanent decrease in bilayer resistance on incorporation of the membrane fractions implies that the fractions also induce ion transport. In general the increase in conductance was approx. 10-fold. We did not find conductances to increase by as larger factor as some of the D-glucose permeability increases. While there is no a priori reason for believing the increases should be comparable, one possible reason for the lower increase in conductance might be that the anion transport system believed to be associated with band 3 may only be partially incorporated into the bilayer. Although significant increases in D-glucose permeability were always observed in the presence of membrane extracts, there does not however, appear to be a clear cut relationship between the protein concentration of the membrane extracts in the bathing solution and the D-glucose permeability although the largest effects observed ($P/P_0 \approx 46$) occurred with relatively high protein concentrations. A possible explanation for this is that the composition of the fractions in particular their detergent content and their extent of aggregation is somewhat variable and this indirectly may well influence the nature and extent of penetration of the complex into the bilayer. Denaturation may also occur during extraction. The problem of variation in activity is not unique to the planar bilayer experiments, Kasahara and Hinkle

[9] found considerable variations from experiment to experiment in the liposome system.

At present it is only possible to tentatively speculate about the reasons why we found that bilayers with one face in contact with solution containing membrane extract were stable whereas we were unable to establish a stable symmetrical system. The principle polypeptide components common to all the fractions are bands 3 and 4.2 and the Triton X-100 complex of these components possibly of overall composition $3_2(4.2)_2$ $(\text{Triton})_{\approx 400}$ has a strong tendency to aggregate. It seems reasonable to assume that it will be the unaggregated species which penetrates the bilayer, most probably with a preferred orientation imposed by the more hydrophilic band 4.2 tetramer and possibly with release of bound Triton X-100. Rupture of the bilayer is most likely to occur at the plateau border between the bilayer and the perimeter of the teflon orifice. It is conceivable that when the complex is present at both faces of the bilayer association between complexes on opposite sides of the bilayer could lead to association within the plateau border region which ultimately results in dewetting of the Teflon orifice and rupture of the bilayer.

The maximum transport activity we have observed ($P = 212 \cdot 10^{-8}$ cm $\cdot$ s^{-1}) corresponds to the facilitated diffusion of $0.38 \cdot 10^{-8}$ mol $\cdot$ min^{-1} $\cdot$ cm^{-2} of the bilayer at 27°C. In the erythrocyte the maximum velocity of transport at 20°C is in the range $0.45 \cdot 10^{-8}$—$1.13 \cdot 10^{-8}$ mol $\cdot$ min^{-1} $\cdot$ cm^{-2} depending on the method of measurement [35] so that in this case the in vitro system is between 30 and 80% as efficient as that of the in vivo system.

On the basis of the present study the trans membrane polypeptides of band 3 would appear to be most likely associated with glucose transport activity in agreement with other published data [9—11,13—15] since band 4.2 polypeptides are located on the cytoplasmic side of the erythrocyte membrane, however we cannot completely eliminate the possibility that relatively minor highly active components of the erythrocyte membrane (e.g. band 4.5 polypeptides) in our extracts could be responsible for the observed effects. The fact that we observed the largest permeability coefficients with the crudest extract is perhaps significant in this context. Furthermore as the composition of the bilayer in the presence of membrane extracts is not known it is also possible that a minor active component in the extract could be preferentially adsorbed into the bilayer. Despite these difficulties the establishment of a facilitated diffusion system in a planar bilayer constitutes a first towards the unambiguous identification of the monosaccharide carrier of the human erythrocyte membrane which should lead to a better understanding of carrier-mediated transport.

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