Glycation of the human erythrocyte glucose transporter *in vitro* and its functional consequences

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Glycation of human erythrocyte membrane proteins was induced by incubation *in vitro* with high concentrations (80 mM or 200 mM) of D-glucose for 3 or 6 days. The extent of glycation was quantified from the covalent incorporation of ^3H by reduction of the glucose glycation products with NaB^3H_4 . For membranes incubated for 3 days with 80 mM-D-glucose, glycation *in vitro* of Band 4.5 (containing the glucose transporter) was equivalent to 0.11 mol of glucose/mol of glucose transporter, compared with ^3H labelling in 3-day-incubated control membranes of 0.055 mol of glucose/mol of glucose transporter. In membranes incubated for 6 days with 200 mM-D-glucose, glycation increased to 0.21 mol of glucose/mol of glucose transporter, whereas the controls without glucose had 0.11 mol of glucose/mol of glucose transporter. Glycation *in vitro* was accompanied by a fall in the B_{max} of binding of ^3H cytochalasin B (a competitive inhibitor of glucose transport), without any change in the binding affinity. The data suggest that glycated glucose transporters have decreased ability to bind cytochalasin B. It is proposed that glycation can alter glucose transporter activity.

INTRODUCTION

Glycation, also known as non-enzymic glycosylation, is the condensation reaction between carbohydrate and protein amino groups. The reaction of glucose with amino groups of proteins results in the formation of a stable oxoamine glucose adduct (Amadori product) through the rearrangement of an aldimine Schiff base [1–3]. The reactive amino groups can be either the $\alpha\text{-NH}_2$ group of the protein *N*-terminus or $\epsilon\text{-NH}_2$ groups of certain lysine residues, depending on their accessibility and environment [4–6]. Glycation has been found to occur both *in vivo* and *in vitro* [7]. This reaction is responsible for the formation of the glycosylated (or glycated) haemoglobins and is the cause of the peculiar chromatographic behaviour of haemoglobin A_{1c} [4]. The extent of haemoglobin A_{1c} accumulation depends on the concentration of glucose in the plasma during the preceding weeks (up to 17 weeks, the lifetime of the human erythrocyte). This property has been used successfully as an index of diabetic control [8]. In addition to haemoglobin, other long-lived proteins have been found to undergo glycation *in vivo*, such as lens crystallins [9], collagen [10], low-density lipoprotein [11], albumin [5], fibronectin [12], erythrocyte membrane proteins [13], bone osteocalcin [6], and myelin [14].

Glycation can markedly affect protein function. For example, glycation of the two β -chain *N*-terminal amino groups that are involved in the binding of 2,3-disphosphoglycerate leads to increased oxygen affinity [15]. Also, glycation of lens crystallin induces a conformational change which increases SH-group exposure to oxidation [16]. In addition, the extent of glycation of functional lysine residues of ribonuclease A is paralleled by increased inactivation of the enzyme [17].

The human erythrocyte is enriched in glucose transporters, the proteins that facilitate the translocation of D-glucose across the plasma membrane [18,19]. The human erythrocyte glucose transporter constitutes a large fraction of a group of polypeptides, Band 4.5, which span an M_r range of 66000 to 45000 on SDS/PAGE [20]. The diffuse nature of Band 4.5 is due to

heterogeneous biosynthetic glycation [21,22]. Endoglycosidase F treatment of Band 4.5 decreases biosynthetic glycation by removal of *N*-linked oligosaccharide moieties, resulting in a sharper glucose transporter protein band on SDS/PAGE, detected both immunologically [22], and functionally through photolabelling with cytochalasin B [23]. This agent is a potent inhibitor of carrier-mediated D-glucose transport ($K_i \sim 10^{-7}$ M). Cytochalasin B binds to the inward-facing glucose-binding site since it is a competitive inhibitor of glucose efflux [24]. D-Glucose-displaceable cytochalasin B binding to erythrocyte membranes has a K_d of 10^{-7} M and a B_{max} of 550 pmol/mg. Furthermore, cytochalasin B binding to the glucose transporter exhibits a one-to-one stoichiometry [20].

The amino acid sequence of the erythrocyte transporter is highly similar to that derived from a cDNA clone of the human Hep G2 hepatoma glucose transporter [25]. Hydropathy analysis of the transporter amino acid sequence suggests that this protein contains 12 membrane-spanning helices [25]. Of these, helices numbers 7–10 are proposed to be involved in glucose transport across the lipid bilayer [26].

Given the long life span of human erythrocytes and the lack of protein renewal mechanisms in these cells, erythrocyte proteins can undergo significant glycation *in vivo*. Moreover, a lysine residue appears to be essential for cytochalasin B binding and sugar transport [27,28]. Hence it is possible that the erythrocyte glucose transporters are susceptible to glycation and consequently altered function. In this study we show that the erythrocyte glucose transporter can be glycated *in vitro* and, further, we examine the effects of glucose transporter glycation on cytochalasin B binding.

EXPERIMENTAL

Materials

D- and L-glucose, cytochalasins B and E, NaN_3 , phenylmethanesulphonyl fluoride (PMSF), E-64 [$[N\text{-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane}$;

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; E-64, [$N\text{-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane}$; BCIP, 5-bromo-4-chloro-indoylphosphate-*p*-toluidine salt; NBT, NitroBlue Tetrazolium; DTT, dithiothreitol.

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oxiran-2-carbonyl)-L-leucyl]-amido-(4-guanido)butane), EGTA, sodium dibasic and monobasic phosphate, SDS and Hepes were obtained from Sigma. [^3H]Cytochalasin B (22 Ci/mmol) was from Amersham. NaB^3H_4 (1400 mCi/mmol) was from ICN. Nonidet P-40 was from Calbiochem. Protein A-Sepharose CL-4B was from Pharmacia. Sodium deoxycholate, dimethyl formamide, Tween 20 and D-glucose were from Fisher. BCIP (5-bromo-4-chloro-indoylphosphate-*p*-toluidine salt), NBT (NitroBlue Tetrazolium) and goat anti-rabbit-alkaline phosphatase conjugate were from Bio-Rad. Tris base and endoglycosidase F were from Boehringer Mannheim. Outdated blood was obtained from the Hospital for Sick Children blood bank.

Preparation of erythrocyte membranes

Outdated blood was suspended in 10 vol. of phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.4), followed by centrifugation at 3500 *g* for 5 min at 4 °C and collection of erythrocytes. This procedure was repeated three times. Washed erythrocytes were lysed by mixing with 20 vol. of 5 mM-sodium phosphate, pH 8.0. The erythrocyte membranes were collected by centrifugation at 31000 *g* for 15 min at 4 °C. The membranes were washed free of haemoglobin by resuspension and centrifugation at least four more times in 5 mM-sodium phosphate, pH 8.0 [29].

Purification of Band 4.5

The method used was essentially that of Baldwin *et al.* [20]. Erythrocyte membranes (4 mg of protein/ml) were stripped of peripheral proteins by mixing with 15.4 mM-NaOH/2 mM-EDTA/0.2 mM-dithiothreitol (DTT) for 10 min. The membranes were immediately centrifuged at 48000 *g* for 15 min at 4 °C and the pellets were resuspended in 50 mM-Tris/HCl, pH 6.8. The membranes were centrifuged again and the pellets were resuspended in 50 mM-Tris/HCl/2 mM-DTT, pH 7.4, to a final protein concentration of 2 mg/ml. Solid octyl glucoside was added to a final concentration of 46 mM and the membranes were shaken for 20 min at 4 °C. The membranes were centrifuged at 130000 *g* for 1 h. A sample of the supernatant (30 ml) was applied to a 2.5 cm $\times$ 6.3 cm column of DE-52 equilibrated with 34 mM-octyl glucoside in 50 mM-Tris/HCl/2 mM-DTT, pH 7.4. The column was eluted with the column equilibration buffer at 70 ml/h, and 2 ml fractions were collected. Protein-containing fractions (determined by the Bradford procedure [30]) were pooled. This material, which contained the glucose transporter and erythrocyte phospholipids, was dialysed against 4 $\times$ 2 litres of 50 mM-Tris/HCl/1 mM-EDTA/100 mM-NaCl, pH 7.4, over a period of 48 h. The lipid vesicles containing glucose transporters were recovered by centrifugation at 240000 *g* for 1 h. The pellet was resuspended to a protein concentration of approx. 4 mg/ml.

Production of anti-(Band 4.5) antiserum

Antibodies to the erythrocyte Band 4.5 were raised in two New Zealand White rabbits (male, 1.5–2 kg) by repeated injection of 250 μg of purified Band 4.5 according to the method of Baldwin & Lienhard [31]. Both rabbits were bled (10 ml), before any injections with antigen, to prepare preimmune sera. The initial injection was a 1:1 mixture of Freund's complete adjuvant and antigen. All subsequent injections were a 1:1 mixture of Band 4.5 with incomplete Freund's adjuvant. Booster injections were given every 3 weeks. Samples of blood (8 ml) were taken from both rabbits 10 days after each booster injection. The blood was allowed to clot for 2 h at 4 °C and was then centrifuged to collect the antiserum supernatant. Antiserum was stored at –20 °C before use.

Western blot analysis

Membrane samples (30 μg) were separated by SDS/PAGE (5% stacking gel, 12% separating gel) according to Laemmli [32]. Gels were run at 200 V in a Bio-Rad Mini Protean II gel apparatus. Gels were incubated with transblot buffer [25 mM-Tris base/192 mM-glycine/20% (v/v) methanol, pH 8.3] for 30 min before transferring the proteins on to nitrocellulose paper for 2 h at 100 V. The nitrocellulose was incubated for 1 h in blocking buffer (3% skim milk powder/0.05% Tween 20/150 mM-NaCl/50 mM-Tris/HCl, pH 7.5) at room temperature, then incubated overnight in blocking buffer, containing antiserum at a 1:500 dilution at 4 °C. The nitrocellulose was then washed with three 15 min washes of blocking buffer and incubated for 1 h with goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) at a 1:3000 dilution in blocking buffer. Following three 15 min washes, the nitrocellulose was incubated with the colour-developing reagent (4 mM-MgCl₂, 0.1 mg of NBT/ml and 0.05 mg of BCIP/ml in 100 mM-Tris/HCl, pH 9.2). The reaction was stopped with cold water after 20 min.

Glycation of erythrocyte membranes *in vitro*

Glycation of erythrocyte membranes *in vitro* was carried out by incubating membranes at 1 mg of protein/ml with 80 mM- or 200 mM-D-glucose (where indicated) in 5 mM-sodium phosphate, pH 8.0, containing 3.1 mM-Na₂S₂O₈, 0.1 mM-PMSF, 0.1 mM-EGTA and 0.01 mM of the thiol proteinase inhibitor E-64, for 3 days at 37 °C with mixing. Control membranes were incubated in parallel in the absence of D-glucose, but glucose was added at the end of the incubation at concentrations matching those of the glycated samples. Control and glucose-incubated membranes were immediately washed free of D-glucose by centrifugation at 31000 *g* for 20 min at 4 °C, and this step was repeated three times with homogenization of the pellet between centrifugation steps. The membranes were resuspended to protein concentrations of 2–3 mg/ml in 5 mM-sodium phosphate, pH 8.0, and stored at –120 °C.

Measurement of glycation

^3H -labelling of glycation sites. The degree of glycation was determined by the method of Bookchin & Gallop [33] as modified by Miller *et al.* [13], based on the permanent incorporation of ^3H into the Schiff base and oxoamine products of glycation upon reduction with NaB^3H_4 . Briefly, a 100-fold molar excess of NaB^3H_4 (1400 mCi/mmol) over protein was used, assuming an average M_r of 100000 for membrane proteins. The membranes were concentrated to 7 mg of protein/ml in a Hettich Microfuge at 11000 *g* for 10 min at 4 °C, NaB^3H_4 was added to a final concentration of 7 mM and the mixture was incubated for 40 min at room temperature. Unreacted NaB^3H_4 was washed away by dilution with 10 vol. of 5 mM-sodium phosphate, pH 8.0, followed by two centrifugation steps at 11000 *g* for 10 min at 4 °C and two centrifugations with 200 vol. at 31000 *g* for 20 min at 4 °C. NaB^3H_4 adds a stable radioactive label to the carbon chain of the aldimine and oxoamine glycation products forming hexitol-lysine derivatives. Since one ^3H atom (or H atom) is incorporated per glycation product, the amount of radioactivity incorporated into protein is a measure of glycation. Radioactivity incorporated specifically into Band 4.5 was determined by endoglycosidase F treatment of membranes followed by SDS/PAGE and gel slicing or by immunoprecipitation with an anti-(Band 4.5) rabbit serum.

Separation of ^3H -labelled, endoglycosidase F-treated, Band 4.5. ^3H -labelled membranes (25 or 30 μg of protein) in 5 mM-sodium

phosphate, pH 8.0, were taken to dryness in a Speed-vac. The dry residue was resuspended by agitation and sonication in 40 μ l of incubation buffer (250 mM-sodium acetate, 20 mM-EDTA, 10 mM- β -mercaptoethanol, 0.1 mM-PMSF, 0.01 mM-E-64 and 3.1 mM- NaN_3 , pH 6.2). Endoglycosidase F (0.5 units, 10 μ l) was added to each membrane sample, and the tubes were capped and incubated at 37 °C with rotation for 18 h. A 50 μ l portion of SDS/PAGE sample buffer (0.25 M-Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 50 mM-DTT and Bromophenol Blue) was then added to each sample, and proteins were separated by SDS/PAGE and stained with Coomassie Blue. The samples lanes were sliced into 2 mm slices, digested with 0.75 ml of 30% H_2O_2 at 60 °C for 3 h or more and radioactivity was determined by scintillation counting in 10 ml of scintillation cocktail. Addition of detergents (0.5% Triton X-100 and 0.05% SDS) to the endoglycosidase F incubation, as described by Lienhard *et al.* [22], was omitted, since it did not increase the apparent efficiency of the reaction, and furthermore led to the formation of high molecular aggregates of the glucose transporter as detected by Western blotting (results not shown).

Immunoprecipitation of ^3H -labelled Band 4.5. A 100 μ g portion of ^3H -labelled membranes at 2 mg of protein/ml was solubilized with 1 vol. of 5% Nonidet P-40/2% sodium deoxycholate/330 mM-EDTA/50 mM Tris/Cl (pH 7.4)/5% SDS (buffer A) to 4 vol. of membrane suspension, and PMSF (0.5 mg/ml) and pepstatin (0.02 mg/ml) were added. The samples (60 μ l) were mixed by rotation for 30 min at 4 °C and then diluted 9-fold with 480 μ l of 1% Nonidet P-40/0.4% sodium deoxycholate/66 mM-EDTA/50 mM-Tris/Cl (pH 7.4) (buffer B). Antiserum or pre-immune serum (60 μ l) was added to the diluted samples, so that they were diluted 10-fold and the final concentration of SDS was 0.1% (w/v). The samples were incubated overnight at 4 °C and then 150 μ l of Protein A-Sepharose CL-4B beads, suspended in phosphate-buffered saline + 0.1% NaN_3 at 20% (v/v), was added (in a 3-fold excess). The amount of Protein A-Sepharose beads used was based on the assumption that 1 μ l of swollen beads binds 10 μ g of rabbit IgG, and that serum contains 20 μ g of IgG/ μ l. After incubation for 2 h at room temperature with mixing, the beads were pelleted by a 20 s high-speed spin in a Hettich Microfuge. The supernatants were discarded and the pellets were washed once with 1 ml of buffer B containing 0.5% SDS and twice with 1 ml of phosphate-buffered saline. After centrifugation, the bottom of each Microfuge tube was cut off into scintillation vials. Scintillation cocktail (10 ml) was added and the samples were counted for ^3H radioactivity. Samples containing preimmune serum in place of antiserum were incubated in parallel to measure non-specific immunoprecipitation. The ^3H radioactivity measured in the preimmune serum samples was subtracted from the ^3H radioactivity measured for parallel antiserum samples.

Acetone and trichloroacetic acid protein precipitation

^3H -labelled membranes (10 mg of protein) were mixed with 50 μ l of a 10 mg/ml solution of BSA and 40 μ l of unlabelled erythrocyte membrane (4 mg/ml). Protein was precipitated with 1.0 ml of ice-cold acetone. The samples were mixed by rotation for 1 h and the precipitate was pelleted by centrifugation. The pellets were solubilized with 10% SDS, the supernatants were transferred to scintillation vials and the acetone was allowed to evaporate before adding scintillation cocktail.

Trichloroacetic acid precipitations were performed according to Peterson [34]. Precipitates were washed twice with 0.5 ml of diethyl ether to remove residual trichloroacetic acid. Precipitates were resuspended in 5 mM-sodium phosphate, pH 8.0, containing 1% SDS.

Equilibrium binding of cytochalasin B

D-Glucose-inhibitable cytochalasin B binding to erythrocyte membranes was measured as described by Lienhard *et al.* [27]. Briefly, 50 μ g samples of membrane protein in 5 mM-sodium phosphate, pH 8.0, were incubated with various concentrations (0.025–1 μM) of [^3H]cytochalasin B in parallel samples containing 200 mM-D- or L-glucose, 0.1 mM-PMSF and 5 mM-Hepes, pH 7.4. Cytochalasin E (5.5 μM) was added to each sample to decrease binding of [^3H]cytochalasin B to membrane components other than the glucose transporter. The final sample volumes were 60 μ l. Following centrifugation at 100 000 g in a Beckman 42.2 Ti rotor for 1 h at 4 °C, the supernatants and membrane pellets were separated, and the radioactivity in each fraction was determined as free and bound [^3H]cytochalasin B respectively. All determinations were performed in triplicate with each D- or L-glucose sample. When the cytochalasin B binding data for each membrane sample were plotted in the bound/free versus bound Scatchard form, the non-specific binding data (determined in the presence of D-glucose) fitted a negative single exponential curve. The data points for total binding (determined in the presence of L-glucose) also formed a negative exponential curve that was subtracted from the non-specific curve along radial lines drawn from the origin through the 'total binding' points. The radial subtraction yielded a linear L-glucose minus D-glucose curve which was fitted by linear regression. Protein measurements were according to Lowry *et al.* [35].

RESULTS

Glycation of erythrocyte membranes

Membranes were incubated for 3 days at 37 °C with or without 80 mM-D-glucose as described in the Experimental section. After the incubation, the membranes were washed and treated with NaBH_4 to label the glycation sites. The extent of glycation was determined by treating the membranes with endoglycosidase F followed by SDS/PAGE and gel slicing. Fig. 1(a) shows that 0.5 unit of endoglycosidase F is necessary to remove the biosynthetic glycosylation from Band 4.5 polypeptides present in 25 and 30 μ g membrane samples. Band 4.5 was detected by Western blotting. Removal of the N-linked oligosaccharide from purified Band 4.5 allowed it to migrate in SDS/PAGE as a narrower band of an average M_r of 41 000.

Purified deglycosylated Band 4.5 migrated as a doublet (upon close examination) at an M_r of 42 000 to 40 000, as revealed by Coomassie Blue staining of the SDS/polyacrylamide gel in Fig. 1(b) (lane GT). The Band 4.5 doublet separated away from the other erythrocyte membrane polypeptides (lanes 0.2–0.5). This region was excised and prepared for scintillation counting as described in the Experimental section. Care was taken not to include the thin distinct band at an M_r of 43 000 in the gel slices. It should be stated that endoglycosidase F cannot remove the NaBH_4 -reduced hexitol derivative of glucose from proteins.

Table 1 shows the extent of Band 4.5 glycation in endoglycosidase-F-treated membranes preincubated under different experimental conditions. Table 1 expt. A demonstrates that a 3 day incubation with 80 mM-glucose caused a 1.8-fold increase in the ^3H incorporated into Band 4.5/ μ g of membrane protein, relative to Band 4.5 from membranes incubated for 3 days without glucose (control). Thus incubation of erythrocyte membranes for this period of time with a high concentration of glucose resulted in a significant increase of Band 4.5 glycation ($P < 0.05$, two-tailed *t*-test). Experiments were carried out to verify that there was no carried-over glucose that could interfere with the NaBH_4 reaction. For this purpose, membranes were exposed to 0, 80 and 200 mM-glucose, and immediately washed

Fig. 1. SDS/PAGE of erythrocyte membranes treated with various amounts of endoglycosidase F

Membrane samples (30 μ g) were incubated with the indicated amounts of endoglycosidase F and then subjected to SDS/PAGE. GT is purified Band 4.5 that was also treated with endoglycosidase F. GT served as a standard to locate the glucose transporter in the membrane samples. (a) Western blot with anti-(Band 4.5) serum. The positions of the M_r markers are indicated. (b) Coomassie Blue staining of an SDS/polyacrylamide gel with erythrocyte membranes treated with various concentrations of endoglycosidase F. The five prominently stained bands in the lane marked M represent M_r protein markers of 97 000, 66 000, 43 000, 31 000 and 21 500 (from top to bottom). GT and EF indicate the positions of the glucose transporter and the main endoglycosidase F protein bands respectively.

Table 1. Glycation *in vitro* of Band 4.5 in erythrocyte membranes

Glycation was measured as ^3H incorporated (d.p.m.) into Band 4.5 after reduction of the glucose adducts with NaB^3H_4 , followed by endoglycosidase F treatment and separation of the Band 4.5 by SDS/PAGE as described in the Experimental section. Expt. A: Band 4.5 glycation levels in membranes incubated without (control) or with 80 mM-glucose for 3 days (glycated). The data are the means $\pm$ S.D. from two different sets of control and glycated membranes. Expt. B: Band 4.5 glycation in unincubated membranes and membranes incubated for 3 days without glucose. Expt. C: Band 4.5 glycation levels in control membranes incubated for 6 days without (control) or with 200 mM-glucose (glycated). Expt. D: Band 4.5 glycation levels in erythrocyte membranes ^3H -labelled with NaB^3H_4 followed by immunoprecipitation of Band 4.5 as described in the Experimental section. Membranes were incubated for 3 days without (control) or with 80 mM-glucose (glycated).

Experiment	Membranes	Glycation (d.p.m./ μ g of protein)
A	Control	99 $\pm$ 19
	Glycated	176 $\pm$ 31
B	Unincubated	41 $\pm$ 13
	Incubated	76 $\pm$ 4
C	Control, 6 days	194 $\pm$ 12
	Glycated, 6 days	364 $\pm$ 17
D	Control	129 $\pm$ 1
	Glycated	362 $\pm$ 62

and centrifuged. These membranes were then exposed to NaB^3H_4 and immediately washed and centrifuged as performed with the experimental samples. There was no effect on the amount of NaB^3H_4 radioactivity associated with the membrane proteins as a result of prior exposure of the membranes to a range of glucose concentrations for short periods of time (results not shown). This indicates that free glucose was effectively washed away from the membranes before reaction with NaB^3H_4 , and that the differences in ^3H labelling observed between the samples incubated with and without glucose for 3 days (glycated versus control) were due to the chemical reaction of glucose with proteins.

Table 1, expt. B illustrates a different experiment in which a

portion of a membrane sample was treated with NaB^3H_4 immediately after the membrane was prepared from erythrocytes. Another portion of the same sample was first incubated for 3 days under conditions identical to those used for the control in Table 1, expt. A, and subsequently treated with NaB^3H_4 . Both membrane samples were treated with endoglycosidase F and the amount of ^3H radioactivity in Band 4.5 from each sample was determined as described in the Experimental section. The ^3H incorporated into the unincubated membrane sample may represent endogenous glycation which occurs in erythrocytes *in vivo*. The reason for the difference in ^3H -labelling between the unincubated and incubated samples is not totally clear, but it may be due to oxidation of Band 4.5 (which generated NaB^3H_4 -reducible sites) during the incubation process. Outdated blood was used throughout this study to provide erythrocyte membranes. These blood samples were stored in the presence of dextrose for several weeks after collection before their use. Band 4.5 isolated from these samples is already glycated before incubation (see Table 1, expt. B). An experiment was performed to compare the glycation of membranes in these stored samples with that of freshly drawn blood. Membranes prepared from stored and freshly drawn blood were ^3H -labelled and proteins were precipitated with trichloroacetic acid. On average, the radioactivity associated with membrane proteins prepared from outdated blood was 1.3-fold higher than the radioactivity associated with membrane proteins from freshly drawn blood (results not shown). However, this difference should not compromise the difference observed in glycation between control and glucose-incubated samples within the same blood sample.

Table 1, expt. C illustrates the amount of ^3H incorporated into Band 4.5 from membranes incubated without (control) or with 200 mM-D-glucose for 6 days at 37 $^{\circ}\text{C}$. These conditions resulted in a 1.9-fold increase in glycation over the control. Incorporation of ^3H into Band 4.5 from the 6-day-incubated control was greater than that measured in the 3-day-incubated controls of Table 1, expts. A and B. Thus the number of borohydride-reducible sites increased with incubation time in the absence of glucose. Nevertheless, the difference in incorporated ^3H between the control incubated and glucose-incubated membranes represents glycation *in vitro*, and this difference increased, in absolute terms, when exposure to glucose was lengthened from 3 to 6 days.

The stoichiometry of incorporated ^3H to glucose transporter

was calculated to be 0.055 mol of glucose/mol of glucose transporter in control membranes and 0.11 mol of glucose/mol of glucose transporter in membranes glycated for 3 days with 80 mM-glucose (calculated from data in Table 1, expt. A). These values increased to 0.11 mol of glucose/mol of glucose transporter in the 6-day-incubated control and 0.21 mol of glucose/mol of glucose transporter in membranes glycated for 6 days with 200 mM-glucose (see Table 1, expt. B). These calculations were based on (i) an estimate of the number of copies of glucose transporters applied to SDS/PAGE, measured by binding of cytochalasin B in the control membranes (see Fig. 2); and (ii) the fact that one cytochalasin B molecule binds per glucose transporter polypeptide [20]. These values demonstrate that, under the conditions used, glycation occurs on approx. 10–20 % of the glucose transporter molecules, for 3 and 6 day incubations at 80 mM- and 200 mM-glucose respectively.

The amount of glycation of Band 4.5 was also determined by immunoprecipitation of the glucose transporter from ³H-labelled membranes: control-incubated membranes and glucose-incubated (80 mM-glucose for 3 days) membranes were treated with anti-(Band 4.5) antiserum as described in the Experimental section. The results are presented in Table 1 (expt. D) and indicate that the membranes glycated *in vitro* had more glycation than control membranes. In several independent experiments a difference between control and glycated samples was always observed, although significant variation in the amount of ³H incorporated was observed associated with the immunoprecipitate. This raised the possibility that ³H-labelled lipids remaining associated with the Band 4.5 molecules after immunoprecipitation could be the source of the variation. Lipids can react with NaB³H₄ at sites of unsaturation (carbon–carbon double bonds) and at oxidized sites that may have resulted from lipid peroxidation. Therefore the amounts of ³H incorporated into membrane protein from two sets of 3-day-control and 3-day-glycated membranes were determined. Delipidation was achieved by acetone precipitation of membrane protein as described in the Experimental section. The results indicated that 40–50 % of the ³H radioactivity was precipitated with the protein pellet, whereas 50–60 % remained in the acetone supernatant and is therefore probably associated with lipid (Table 2).

When the membranes were immunoprecipitated and analysed by SDS/PAGE, Band 4.5 ran as a broad band, and in addition significant aggregation was noted. Thus it was difficult to account for all of the Band-4.5-associated radioactivity using gel slicing. This precluded the use of SDS/PAGE as a means to first delipidate immunoprecipitated Band 4.5 and then quantify the extent of glycation. In contrast, the diffuse migration of Band 4.5

in SDS/PAGE was not a complication of the endoglycosidase F procedure described in Fig. 1 and Table 1. Therefore those results are considered to better represent the ³H label associated with Band 4.5. Also, these results are not complicated by ³H incorporation into lipid, since the gel front of endoglycosidase-F-treated membrane samples separated by SDS/PAGE contained large amounts of ³H radioactivity (results not shown). Presumably this radioactivity is associated with lipid which migrates in this region during SDS/PAGE.

[³H]Cytochalasin B binding to control and glycated membranes

[³H]Cytochalasin B binding was used to determine whether glycation could affect glucose transporter function. Since cytochalasin B is a highly specific competitive inhibitor and ligand of the glucose transporter, it is a useful tool for probing the integrity of the glucose-binding domain. Fig. 2 compares cytochalasin B binding to membranes incubated without or with 80 mM-D-glucose, in Scatchard analysis form. Glycation decreased the maximum binding (*B*_{max}) compared with control membranes, without altering the *K*_d of binding. Table 3 lists the results of several similar experiments with glycated and control membranes. Glycation for 3 days with 80 mM-glucose typically decreased the amount of cytochalasin B binding by about 26 % without appreciably changing the affinity of cytochalasin B binding. Although expt. D in Table 3 had low *B*_{max} and *K*_d values for both control and glycated membranes, the glycated membranes had a lower *B*_{max} than the controls. In one experiment, cytochalasin B binding to membranes incubated for 6 days at 37 °C with 200 mM-D-glucose had a *B*_{max} of 418 ± 39 pmol/mg, which was significantly different than that for the 6-day-incubated (without D-glucose) control (674 ± 46 pmol/mg). The *K*_d values of cytochalasin B binding to these membranes were 62 ± 12 nM and 92 ± 16 nM respectively. The difference in the *K*_d values was not significant. The errors shown for the cytochalasin B binding parameters were the 95 % confidence intervals. As seen with the samples incubated for 3 days (Table 3), the 6-day-glycated sample had a decreased *B*_{max} relative to controls; however, the percentage decrease was 40 %,

Table 2. Acetone precipitation of NaB³H₄-reduced control and glycated membranes

Control and glycated membranes were incubated for 3 days respectively without and with 80 mM-glucose, and reduced with NaB³H₄ as described in the Experimental section. (a) and (b) represent different membrane samples, each assayed in duplicate. Membrane samples (10 µg of protein) were precipitated with acetone and the supernatants and the pellets were separated for scintillation counting.

Sample		³ H in pellet (d.p.m.)	³ H in supernatant (d.p.m.)
Control	(a)	12020	31 720
	(b)	17410	38 870
Glycated	(a)	27700	52 820
	(b)	26310	42 830

Fig. 2. [³H]Cytochalasin B binding to control membranes and to membranes glycated *in vitro*

Glycated (■) and control (□) membranes were incubated for 3 days at 37 °C with or without 80 mM-glucose respectively. D-Glucose (80 mM) was added to the control sample before both the glycated and control samples were washed by centrifugation in 5 mM-sodium phosphate, pH 8.0. After washing, D-glucose-protectable binding of cytochalasin B was determined as described in the Experimental section. Binding data are presented in Scatchard form.

Table 3. Cytochalasin B binding parameters of control and glycated erythrocyte membranes

A–D represent four independent experiments with membranes incubated for 3 days at 37 °C with 80 mM-glucose (glycated) or without glucose (control). [³H]Cytochalasin B binding was measured as described in the Experimental section. The data were derived from graphs similar to that shown in Fig. 2. Errors represent a 95 % confidence interval.

Experiment	$B_{\max}$ (pmol/mg)		K_d (nM)	
	Control	Glycated	Control	Glycated
A	526 ± 52	385 ± 12	99 ± 20	110 ± 9
B*	497 ± 49	385 ± 15	72 ± 10	82 ± 5
C	566 ± 35	422 ± 48	90 ± 5	79 ± 9
D†	378 ± 39	279 ± 20	62 ± 10	68 ± 11

* These data were taken from the plot in Fig. 2.

† These values are low but exhibit the same characteristics of the other experiments (see the text).

compared with 26 % for membranes glycated for 3 days in 80 mM-glucose.

It is noteworthy that the decrease in cytochalasin B binding exceeds the extent of glycation, in absolute terms, suggesting that this fall may result from a co-operative process between glycated glucose transporters (and/or glycated membrane proteins) and non-glycated glucose transporters, affecting cytochalasin-B-binding properties of non-glycated glucose transporters in the membrane. In addition, the calculation of the percentage glucose transporter glycation may be an underestimate, since the recovery of radioactivity from the endoglycosidase-F-treated samples separated by SDS/PAGE was assumed to be 100 % efficient, but in fact it may not be complete. Further, our calculations assumed that the NaB³H₄ was 100 % pure. It is known that NaB³H₄ decomposes spontaneously in solution (0.01 M-NaOH) to ³H₂O or ³H₂ gas, so that the value of the specific radioactivity of true NaB³H₄ was most probably an overestimate.

In all the incubation experiments *in vitro*, the control samples were mixed with glucose (80 or 200 mM) just before washing and centrifugation (see the Experimental section) in order to compensate for possible non-specific effects of free glucose that may remain with the glycated membranes after the washing steps. Moreover, the effectiveness of the membrane-washing procedure was tested using cytochalasin B binding assay. Membranes were exposed to 0, 80 or 200 mM-glucose and immediately washed and centrifuged as for the experimental samples; cytochalasin B binding was subsequently assayed at 0.49 and 0.79 μ M-cytochalasin B. The results showed no differences among the three samples for either total binding or for D-glucose-protectable binding (non-specific). Total binding at 0.49 μ M-cytochalasin B was 86, 86 and 98 pmol/mg of protein for samples pre-exposed to 0, 80 and 200 mM-glucose, whereas non-specific binding was 70, 69 and 73 pmol/mg respectively. The corresponding values using 0.79 μ M-cytochalasin B binding were 161, 160 and 170 pmol/mg of protein for total binding, and 103, 103 and 113 pmol/mg of protein for the non-specific binding. Hence the decreased cytochalasin B binding observed in the samples glycated *in vitro* results from the time-dependent reaction of glucose with the membranes rather than the non-specific carry-over of free glucose.

The observed decrease in the $B_{\max}$ of cytochalasin B binding in the glycated samples could potentially result from accelerated degradation of the glucose transporter in the membranes incubated with glucose. To test this possibility, the amount of

glucose transporter protein in samples (of equal amounts of protein) was determined by Western blotting using an anti-(Band 4.5) serum. The intensity of the Band 4.5 (M_r 55000) was found to be the same in each sample (results not shown). Further, there were no low- M_r immunoreactive protein bands, indicating that under these conditions of incubation, degradation of the glucose transporter did not occur.

DISCUSSION

Band 4.5 contains both the glucose and the nucleoside transporters. Approx. 95 % of the Band 4.5 polypeptides are glucose transporter, whereas only 5 % are nucleoside transporter [36]. Also, it has been previously shown that all erythrocyte membrane proteins from the blood of diabetic individuals have 2-fold-increased glycation, i.e. no protein shows enhanced increase in glycation compared with another [13]. Glycation of Band 4.5 measured in the present study is probably associated with both transporters since they co-migrate on SDS/polyacrylamide gels after extensive endoglycosidase F treatment similar to that used in this study [37,38]; however, given the prevalence of the glucose transporter in Band 4.5, the majority of the glycation measured is most probably associated with the glucose transporter.

The results in this study indicate that Band 4.5 can be glycated *in vitro*. In control erythrocyte membranes, approx. 5–6 % of the Band 4.5 was found to be naturally glycated, and this value increased to nearly 11 % on exposure *in vitro* to 80 mM-D-glucose for 3 days and to 21 % after exposure to 200 mM for 6 days. Glycation of the Band 4.5 was accompanied by a decrease in the specific binding of cytochalasin B. Therefore it is possible that glycation results in the modification of sites in the glucose transporter that are crucial for cytochalasin B binding and possibly for glucose transport.

Increases in glycation of other proteins have been observed in diabetic individuals with attendant hyperglycaemia. For example, haemoglobin A_{1c} has a glycated β -chain N-terminus [1,33,39]. Normal glycaemia results in glycation of 4–6 % of the total haemoglobin. Diabetic individuals in poor glycaemic control have haemoglobin A_{1c} glycation values of 10 % or more, an increase of 2-fold over normals. Likewise, erythrocyte membrane proteins from diabetic patients have glycation levels some 2-fold above the levels measured in normal individuals [13]. Thus the incubation of normal erythrocyte membranes for 3 days at 80 mM-D-glucose used in this study caused a similar increase in glycation of Band 4.5 to that observed for haemoglobin in diabetic subjects. This glucose concentration and the duration of the incubation are equivalent to the glucose exposure that would occur at 10 mM-glucose for 24 days (this concentration of glucose is not unusual in diabetic individuals).

The results of cytochalasin B binding to membranes glycated *in vitro* indicated that glycation caused a clear decrease in the maximum binding, with little change in the binding affinity. Since cytochalasin B is thought to bind at the cytoplasmic glucose-binding site [24], these results may suggest glycation of a cytoplasmically oriented amino group in cytochalasin B binding and possibly in glucose binding. Like glucose, cytochalasin B has electron-accepting functional groups that are predicted to be important in binding to the glucose transporter and which can interact with electron-donating functional groups such as amino groups [40]. Furthermore, reaction of erythrocyte membranes with the NH₂-modifying reagent 1-fluoro-2,4-dinitrobenzene inhibits cytochalasin B binding [27], and the NH₂-modifying reagent trinitrobenzenesulphonate inhibits sugar transport in erythrocytes [28], lending further support to the involvement of an amino group in glucose transporter function.

In conclusion, we have demonstrated that the erythrocyte glucose transporter can be glycosylated *in vitro*. Furthermore, glucose transporter glycosylation is accompanied by a decrease in its ability to bind cytochalasin B. As stated above, this may reflect a change in the binding of glucose to the transporter and an alteration in glucose transporter function; however, more direct approaches will be required to support this hypothesis. Lastly, it will be of great interest to measure the degree of glucose transporter glycosylation in diabetic individuals *in vivo* and to determine what effects this may have on glucose uptake.

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