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THE STEREOSPECIFIC D-GLUCOSE TRANSPORT PROTEIN IN CHOLATE EXTRACTS OF HUMAN ERYTHROCYTE MEMBRANES

MOLECULAR SIEVE CHROMATOGRAPHY AND ESTIMATION OF MOLECULAR WEIGHT

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Cholate extracts of human erythrocyte membranes (Lundahl, P., Acevedo, F., Fröman, G. and Phutrakul, S. (1981) Biochim. Biophys. Acta 644, 101–107) were fractionated by molecular sieve chromatography on Sepharose 6B, and the size and molecular weight of the active D-glucose transporter were estimated. The eluent contained 10 or 12.5 mM cholate, since higher concentrations inactivated the glucose transporter, and lower concentrations resulted in aggregation. The chromatographic distribution of the transport activity was reproducible, but was broader than one would expect for a homogeneous component. In the presence of 20 mM EDTA and 5 mM dithioerythritol, a combination which affords a highly stable transport activity, a molecular weight of 400 000 ± 20 000 (Stokes' radius 5.9 nm) was estimated for the smallest active component. This value represents an upper limit, since the molecular weight of a non-spherical component would have been overestimated, and since bound cholate was calculated to represent about 12% of the molecular weight. The activity was completely recovered upon rechromatography. In 10 mM EDTA and 10 mM 2-mercaptoethanol, the estimated molecular weight of the smallest active component was 210 000 ± 15 000, and this component was not stable upon rechromatography in 10 mM EDTA and 10 mM 2-mercaptoethanol. In the absence of chelating and reducing agents, cholate extracts from membranes which had been kept for 5 days at 4°C showed three additional active components smaller than 200 000 in molecular weight. Most of the phospholipids eluted later than the active components of molecular weight 400 000 or 210 000, in all experiments. Electrophoretic analysis in dodecyl sulfate of the chromatographic eluents indicates that at least one of the band 3-polypeptides (nomenclature according to Steck, T.L. (1974) J. Cell Biol. 62, 1–19) is a component of the active transporter. This band 3-polypeptide, which we denote 3.3, has an apparent molecular weight of 88 000. The stable transporter of molecular weight 400 000 might be a tetramer of the 3.3-polypeptide. Alternatively, a dimer of the 3.3-polypeptide in complex with lipids might account for this molecular weight. If the 3.3-polypeptide is the transporter subunit and if it binds cytochalasin B with high affinity ($1.8 \cdot 10^5$ sites/cell) the recovered activity per 3.3-polypeptide is around 40% A degradation product of the 3.3-component (possibly a 4.5-component) might account for the unstable active transporter of molecular weight 210 000.

Introduction

We have shown earlier that the glucose transport protein from human erythrocyte membranes can be solubilized in cholate with retention of high activity for about one day [1]. Other workers in this field have isolated an active transporter from Triton X-100 extracts of human erythrocyte membranes. The latter component gives a diffuse band of molecular weight of around 55 000 upon electrophoresis in dodecyl sulfate [2–5]. The apparent molecular size of the complex between the transporter and Triton X-100 is

around 225 000, as estimated from sedimentation and molecular sieve chromatography experiments [6], and a molecular weight of 185 000 for the native transporter in human erythrocytes was estimated from measurements of electron inactivation [7]. Studies on the glucose transport protein from adipocyte plasma membrane in the presence of cholate showed a Stokes' radius between 6 and 8 nm [8].

In order to estimate the molecular size, in the presence of cholate, of the glucose transporter from human erythrocyte membranes, we have done molecular sieve chromatography experiments under conditions where the activity is both high and stable, and also under conditions where the stability is lower. Throughout the work the stereospecific D-glucose transport activity was analyzed after reconstitution into phospholipid liposomes.

The apparent molecular weight of the glucose transport protein in cholate under conditions where the activity is stable corresponds to four times the apparent molecular weight of a band 3-component in dodecyl sulfate electrophoresis (nomenclature according to Steck [9]). This band 3-component, which we denote 3.3, appears in the chromatographic eluate in amounts approximately proportional to the transport activity.

Materials and Methods

Membranes. Human erythrocyte membranes were prepared from fresh blood by the molecular sieve method [10] as described earlier [1,11]. Membranes were immediately frozen in liquid nitrogen and stored at −70°C, unless otherwise specified.

Cholic acid and phospholipids. Cholic acid, egg yolk phospholipids, and radioactive D- and L-glucose were as in Ref. 11. Sepharose 6B and prepacked 1.5 × 5 cm Sephadex G-25 columns as well as most standard proteins used in the calibration of the Sepharose columns were from Pharmacia, Sweden.

Cholate extracts. The extraction was done essentially as in Ref. 1, except that EDTA was added first and cholate last. The final concentrations were: membrane protein, 5 g/l; cholate, 25 mM; NaCl, 200 mM; EDTA, 10 or 20 mM; mercaptoethanol, 10 mM or dithioerythritol, 5 mM.

Reconstitution of the D-glucose transport system. Reconstitution was done essentially as in Ref. 1. For samples containing dithioerythritol 20 mM dithioerythritol was included in the phospholipid solution instead of mercaptoethanol.

Stereospecific D-glucose uptake. The measurements of transport activity were done essentially as in Ref. 1.

K_{av}-values. The scale of elution volume V_e in the chromatographic experiments was expressed as $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_0 is the void volume and V_t the total volume.

Stokes' radius. Stokes' radii were estimated from a diagram of the cube of the Stokes' radii of globular proteins (bovine serum, albumin, aldolase, catalase and ferritin) against their molecular weights.

Acrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to Neville [12] using a pH of 9.18 in the leading buffer of the discontinuous system and a gel concentration T = 11, C = 1.0. The samples were mixed with a solution containing dodecyl sulfate and other components as in Ref. 11. After staining with Coomassie Brilliant Blue R-250 the gels were scanned at 570 nm.

Phospholipid determination. The phospholipid concentration in the chromatography eluates was determined by the method of Bartlett [13]. When the eluent contained phosphate, relative concentrations were obtained by scanning the electrophoresis plates, since, after staining, phospholipids appeared as a zone (cf. Fig. 6 in Ref. 1). The intensity of staining of this zone paralleled the concentration of phospholipids as determined by phosphate analysis (chromatographic experiments, not shown).

Results

Cholate concentration

The glucose transporter from human erythrocyte membranes can be solubilized with 25 mM cholate, whereas higher concentrations cause inactivation [1]. A suitable cholate concentration for the eluant in chromatographic experiments was estimated from a dilution experiment (Fig. 1). The highest activity was found at a final concentration of 10–12 mM cholate. A further test was done by molecular sieve chromatography on a short Sepharose 6B column (Fig. 2). At 25 mM cholate much activity was lost and the residual activity appeared at low elution volumes (Fig. 2a), which suggests that the activity is preserved in aggre-

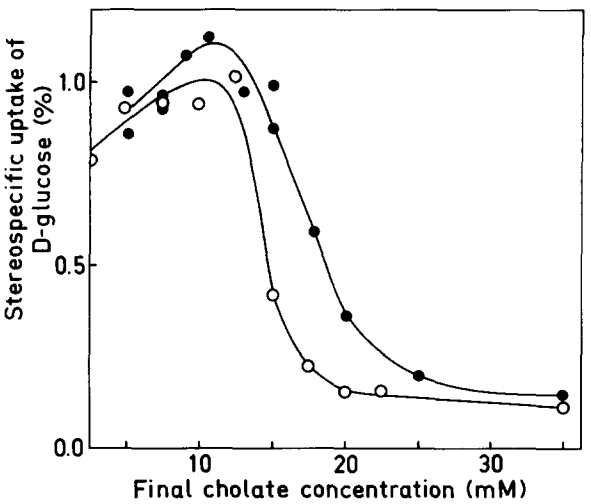


Fig. 1. Dilution of cholate extracts of human erythrocyte membranes to various cholate concentrations. Effect upon stereospecific D-glucose transport activity. Samples of a membrane extract, 25 mM in cholate, were diluted at 4°C with 4 volumes (•———•) or 9 volumes (○———○) of cholate solutions with 200 mM NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 30 mM NaN_3, 5 mM Tris-HCl, pH 8.4, and liposomes were prepared within 15 min following the dilution. The stereospecific D-glucose transport activity was determined in liposomes after reconstitution as described in Materials and Methods. Each point represents the average of two measurements on one batch of liposomes. The activity of the undiluted extract was 2%.

gates of the transporter. At 10 mM cholate (Fig. 2b) the residual activity was 70% of that in a control sample diluted with elution buffer to the same protein concentration as in the pooled eluate. Finally, at 5 mM cholate more aggregates were formed (Fig. 2c). The combined results (Figs. 1 and 2) suggest that a cholate concentration of 10–12.5 mM is suitable for chromatographic fractionation of the membrane extracts.

Molecular sieve chromatography

Chromatographic fractionations were done on Sepharose 6B under three different conditions, based on parallel studies of the stability of the active transporter.

Firstly, membranes were prepared from outdated blood, stored for 5 days at 4°C, and solubilized with 25 mM cholate in 200 mM NaCl and 5 mM Tris-HCl, pH 8.4. Upon chromatography of these extracts in 10 mM cholate activity was detected at $K_{av} \sim 0.3$ and, in addition, three active components eluted at K_{av} values between 0.6 and 1.0 (three experiments, not shown). The polypeptides in the latter fractions might be fragments of the intact transporter and were not found in later experiments with fresh membranes.

Secondly, membranes were prepared from fresh

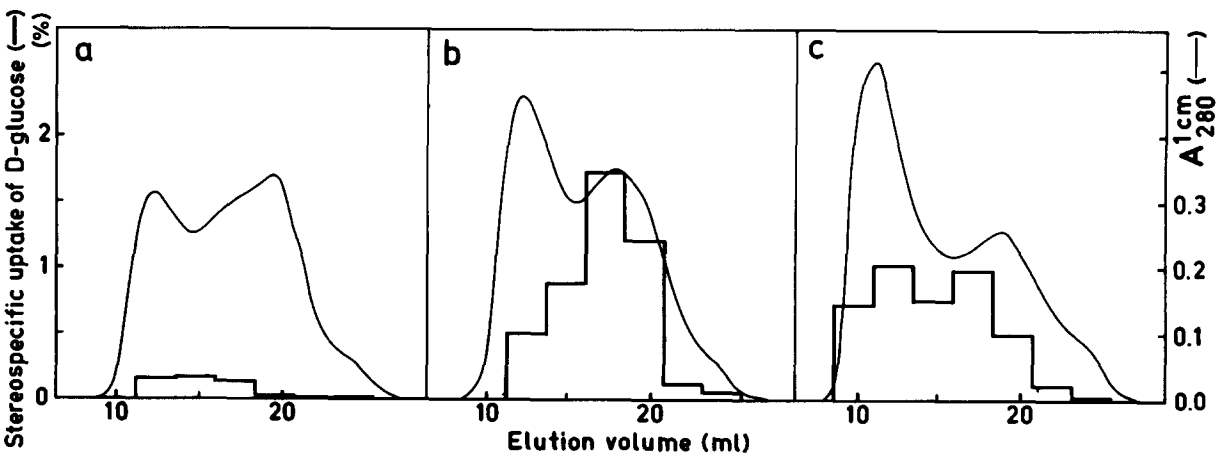
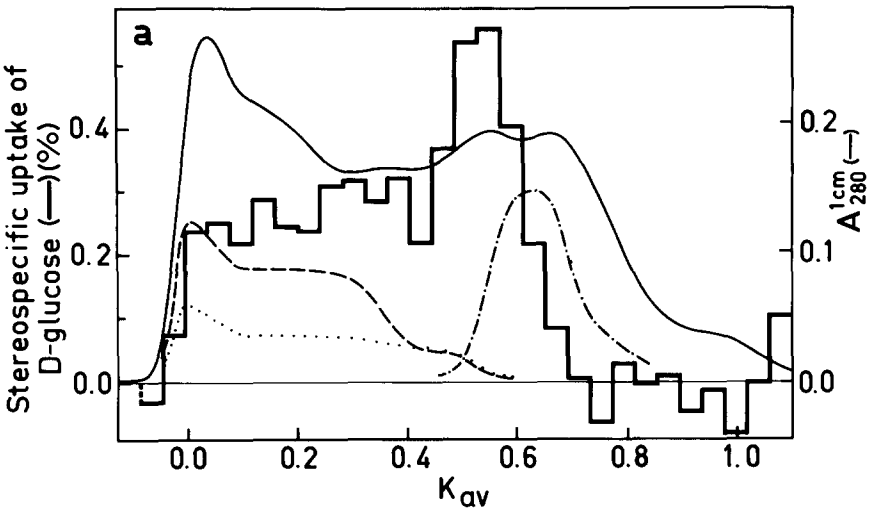


Fig. 2. Molecular sieve chromatography of cholate extracts of human erythrocyte membranes in (a) 25, (b) 10 and (c) 5 mM cholate. A 3-ml sample of 25 mM cholate extract of membranes was applied to a 1.0 × 30 cm Sepharose 6B column and eluted at a flow rate of 2 ml/h at 4°C. The elution buffer contained 200 mM NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 30 mM NaN_3, 5 mM Tris-HCl, pH 8.4, as in the extraction, and 25, 10 or 5 mM cholate. Stereospecific D-glucose transport activity was determined in liposomes after reconstitution, as described in Materials and Methods. Each value represents the average of two measurements on one batch of liposomes.



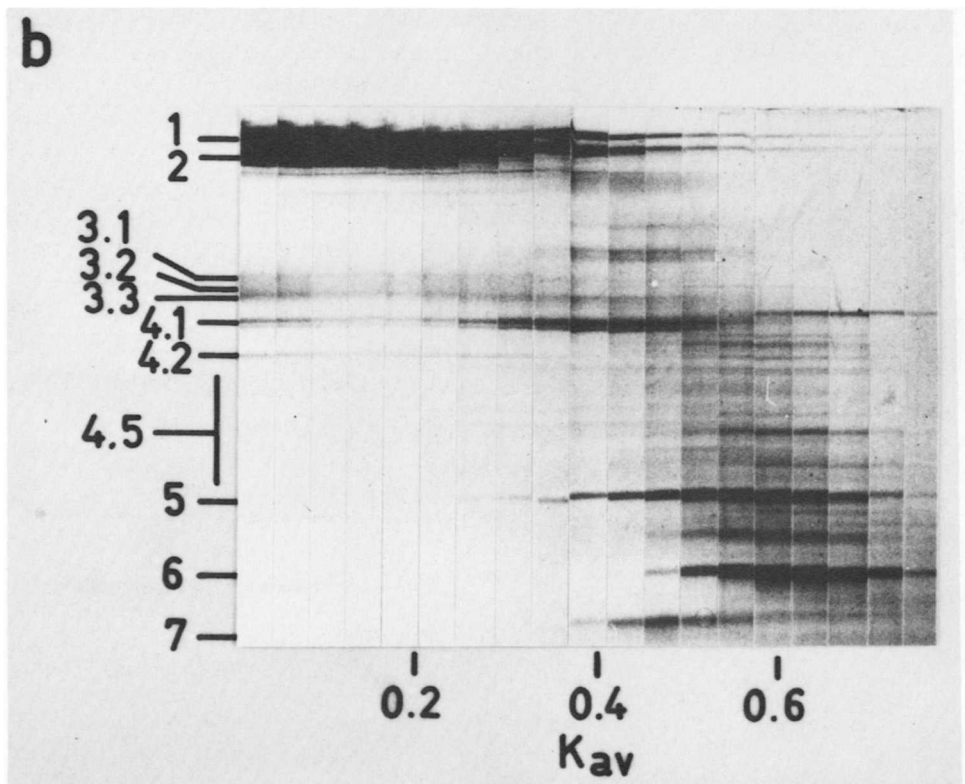


Fig. 3. Molecular sieve chromatography of cholate extract of human erythrocyte membranes. Membranes were extracted with 25 mM cholate in 200 mM NaCl, 10 mM ETDA, 10 mM 2-mercaptoethanol, 30 mM NaN_3, and 5 mM Tris-HCl, pH 8.4. A 5-ml sample was applied to a 1.4 × 72 cm Sepharose 6B column and eluted at 8 ml/h with 10 mM cholate in NaCl, etc., as above, and 5 mM sodium phosphate, pH 8.4. The ordinate scale is expressed as the K_{av} value, defined as $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume, as determined with Dextran 2000 or liposomes and V_t is the total volume as determined with salts. Each determination of transport activity was done in duplicate on one batch of liposomes. (a) Chromatogram showing total band 3-protein (- - - - -) and band 3.3-protein (. . . . .) on an arbitrary scale, and phospholipids (– · – · –), on an arbitrary scale, all as estimated from scans of the electrophoretic patterns (Fig. 3b). (b) Analyses of the eluate by discontinuous acrylamide gel electrophoresis in dodecyl sulfate. The protein bands are denoted according to the nomenclature of Steck [9] except that we have denoted the three main components of the band 3-region 3.1, 3.2 and 3.3. Band 3.3 corresponds to the component of molecular weight 88 000 [9].

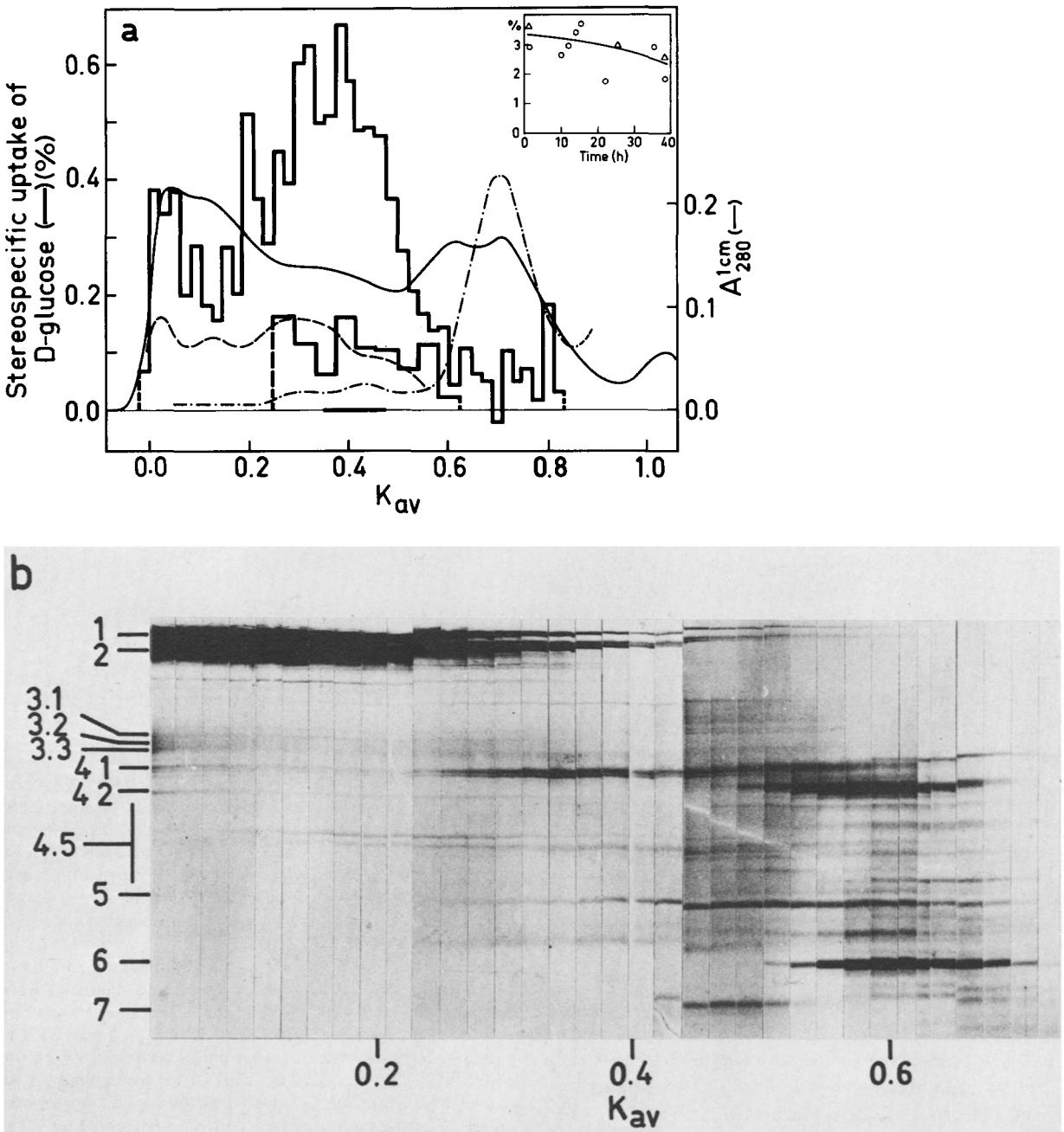


Fig. 4. Molecular sieve chromatography of cholate extracts of human erythrocyte membranes under conditions of improved stability of the glucose transport activity. Membranes were extracted with 25 mM cholate in 200 mM NaCl, 20 mM EDTA, 5 mM dithioerythritol, 30 mM NaN_3, and 5 mM Tris-HCl, pH 8.4. A 20-ml sample was applied to a 3 × 62 cm Sepharose 6B column, and eluted at 31 ml/h with 12.5 mM cholate in NaCl, etc., as above. (a) Chromatogram showing the total amount of band 3 (- - - - - -) on an arbitrary scale, as estimated from scans of the electrophoretic patterns (Fig. 4b), and phospholipids determined as phosphate (– · – · –) on an arbitrary scale. The insert (o———o) shows the decrease in transport activity in the extract with time. The activity peak at K_{av} = 0.43 eluted at about 12 h in the scale of the stability diagram. The triangles show the result of a control experiment (duplicate determinations) on a separate extract. The rechromatographed material (see below) eluted at 31 h. A 20-ml sample of the eluant with K_{av} values from 0.36 to 0.47 as indicated by the bar was immediately rechromatographed (lower histogram of activity distribution). (b) Electrophoretic analyses done as in Fig. 3b. The intense band near 4.2 at $K_{av} \sim$ 0.58 possibly corresponds to a dimer of glyceraldehyde-3-phosphate dehydrogenase.

blood, immediately frozen in liquid nitrogen, stored at −70°C [1], and solubilized with cholate in the presence of 10 mM EDTA and 10 mM 2-mercaptoethanol. The elution buffer was as in the extraction mixture but contained 10 mM cholate. A major fraction of glucose transport activity eluted with a K_{av} value of 0.537 ± 0.007 (two experiments). One of these experiments is illustrated in Fig. 3. About half of the activity eluted at K_{av} values from 0 to 0.5, and very little at K_{av} higher than 0.7. The glucose transporter from human erythrocyte membranes has been suggested to be composed of polypeptides that appear in band 3 [14–16] or region 4.5 [2–6] in dodecyl sulfate gel electrophoresis, and peripheral proteins [9] can be removed with little loss of transport activity (cf. Refs. 17,18). The activity in the experiment of Fig. 3 seems to elute independently of peripheral proteins as expected, and also independently of most of the phospholipids, whereas the elution of band 3-polypeptides paralleled the activity for $K_{av} < 0.5$ (Fig. 3b). The band 3-region in the dodecyl sulfate gel electrophoresis of the eluate shows two distinct bands and one intermediate diffuse band (3.1, 3.3 and 3.2, respectively). The total amount of band 3- and of band 3.3-polypeptides as estimated from scanning of the polyacrylamide gels is indicated in Fig. 3a. Probably, polypeptides in the bands 3.1–3.3 correspond to the active transporter which eluted at $K_{av} < 0.5$. The high activity peak at $K_{av} = 0.537$ can be attributed only partly to band 3-polypeptides. Possibly some components from the 4.5-region (cf. Refs. 2–6) are responsible for most of the transport activity at this K_{av} value, as judged from the relatively low amount of band 3-components and the amount of 4.5-components shown by electrophoretic analyses. The amount of polypeptides from the 4.5-region is very low at K_{av} values <0.5, but a diffuse (cf. Refs. 2–5) 4.5-component might escape detection in the present experiment. When a sample of the eluate, K_{av} from 0.48 to 0.57, was rechromatographed under the same conditions, the recovered activity was nearly 0 and eluted at lower K_{av} values.

Thirdly, since degradation of band 3-polypeptides might account for the appearance of some 4.5-polypeptides [14–16] we made chromatographic experiments as in Fig. 3, but in the presence of 20 mM EDTA and 5 mM dithioerythritol, which favours a higher stability of the transport activity [1,19]. In this case (Fig. 4) no major activity peak was found at K_{av} around 0.54, the overall activity was higher and upon rechromatography of a sample of the eluate, K_{av} from 0.36 to 0.47 (Fig. 4a), the activity eluted at about the same K_{av} values as in the original chromatography (Fig. 4) and the recovery was quantitative.

The amount of band 3-polypeptides paralleled the activity peak as shown by the electrophoretic scanning data represented in Fig. 4a. The amounts of the 3.1- and 3.2-components are very low at K_{av} higher than 0.4, whereas the 3.3-component is visible up to $K_{av} \sim 0.57$ (Fig. 4b). The two sharp bands in the 4.5-

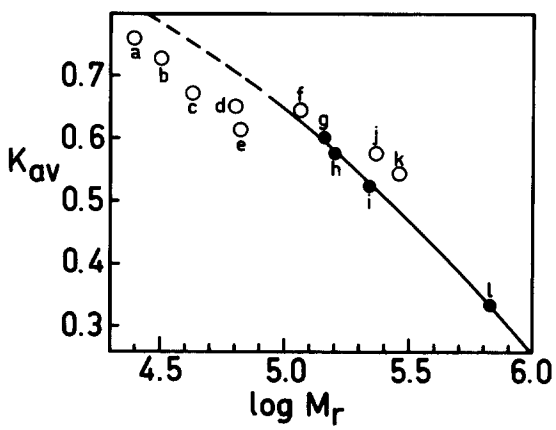


Fig. 5. Calibration of the Sepharose 6B columns. Globular proteins of known molecular weights were used to calibrate the columns. In cases where no dissociation into subunits occurs, the K_{av} values were the same with and without cholate. Most calibration points were determined both in the absence and in the presence of cholate, at pH 7.4 or 8.4 in 200 mM NaCl. The following proteins were used (molecular weights within brackets). (a) Chymotrypsinogen *a* (25 000), (b) half-hemoglobin (32 000), (c) ovalbumin (43 000), (d) hemoglobin (64 000), (e) serum albumin (67 000), (f) half-catalase (116 000), (g) glyceraldehyde-3-phosphate dehydrogenase (144 000), (h) aldolase (158 000), (i) half-ferritin (220 000), (j) catalase (232 000), (k) phycoerythrin (290 000), (l) thyroglobulin (669 000). Open circles, one to three determinations, filled circles, four to six determinations. The average values are plotted. The dashed curve is drawn to approach $K_{av} = 1.0$ as log M_r approaches 3. In our size estimations the portion of the curve between points f and 1 was used. The calibration of the columns for the experiments of Figs. 3 and 4 coincided. The average standard error of the K_{av} values was 0.005 and the range 0.001–0.013. The radius of the points in the figure corresponds to $K_{av} = 0.01$. The K_{av} for glyceraldehyde-3-phosphate dehydrogenase (g) was determined from the scanning of the gels (Figs. 3b, 4b and three additional chromatograms).

region which appeared at low K_{av} values were not seen in previous experiments. The activity of the extract was constant during the time of the chromatography (Fig. 4a, insert, cf. Ref. 19). Electrophoretic analyses in dodecyl sulfate of samples of the eluate kept for one week at 4°C did not differ from those done immediately after the chromatographic experiment, which suggests that little or no proteolytic degradation had taken place.

Estimation of the size of the active transporter in cholate

The chromatographic distribution was broader than one would expect for a homogeneous component (Figs. 3a and 4a). Therefore, the transporter seems to appear in several states, possibly including dimers or tetramers, or complexes with other components. In Fig. 4, the smallest component elutes with a K_{av} value of 0.437 ± 0.008 (two experiments) as estimated from the half-height width of 0.14 K_{av} units which we have found for homogeneous components (aldolase, glyceraldehyde-3-phosphate dehydrogenase and others) with a K_{av} value about 0.4 in the same gel bed and under the same conditions as in Fig. 4. The K_{av} value 0.437 corresponds to a molecular weight of 400 000 ± 20 000 (Stokes' radius 5.9 nm) according to the calibration curve (Fig. 5). The main activity peak in two experiments such as that illustrated in Fig. 3 had a K_{av} value of 0.537 ± 0.007, which corresponds to a molecular weight of 210 000 ± 15 000.

Discussion

To minimize the risk of proteolytic degradation we have prepared membranes from freshly drawn blood and stored them at −70°C. For the same reason we have, in the present work, avoided any treatment of the membranes, such as removal of peripheral proteins, prior to cholate extraction, and we have used EDTA and 2-mercaptoethanol or dithioerythritol during the extraction and fractionation to improve the stability of the active component [1,19]. The cholate concentration is an important factor for the stability of the transporter (Figs. 1 and 2, cf. Refs. 1 and 19). The optimal cholate concentration of 12 mM determined in the dilution experiment illustrated in Fig. 1 suggests that in the extract the non-bound cholate might be about 12 mM.

A higher concentration causes loss of activity due to denaturation of the transporter (cf. Refs. 1, 11 and 19). Similarly, Carter-Su and co-workers [8] have reported that the glucose transporter from rat adipocytes is inactivated upon prolonged exposure to 2% (48 mM) cholate, and that the activity was stable at 0.5% (12 mM) cholate. However, we have found that the solubilized transporter from human erythrocyte membranes is inactivated to less than 20% of the initial activity in 15 min at 4°C after 5–10-fold dilution to a cholate concentration higher than 25 mM (Fig. 1). Probably the mechanism of inactivation by cholate is similar for these two glucose transporters.

Our size estimate of the active transport component in cholate with 20 mM EDTA and 5 mM dithioerythritol gave a Stokes' radius of 6 nm, and a molecular weight of 400 000. This component was stable upon rechromatography, with complete recovery of the activity. The active component of a size corresponding to a molecular weight of around 210 000 was unstable. The activity diminished to nearly zero upon rechromatography in 10 mM EDTA and 10 mM 2-mercaptoethanol. Carter-Su and co-workers [8] have estimated the Stokes' radius of the glucose transporter in the adipocyte membrane at 6-8 nm. The corresponding active material is probably inhomogeneous and the broad activity distribution resembles that in Fig. 4.

The molecular weight estimations are correct if the active component is spherical. Otherwise the estimated molecular weights are too high. In addition the active components might contain lipids (cf. Fig. 4a) and bound cholate. If a spherical transporter of 6 nm radius is surrounded by an annulus of cholate corresponding to the thickness of the lipid bilayer of the membrane, i.e., two cholate molecules deep, the amount of cholate bound will correspond to a molecular weight of 47 000 (size and volume data for cholate from Ref. 20). Therefore, the active transporter has an apparent molecular weight of about 350 000. At most a tetramer of a band 3-component can be accomodated in this size range. Under the conditions of the experiment illustrated in Fig. 3 the major activity peak corresponded to a molecular weight of around 210 000. The 210 000-component might be a tetramer of a 4.5-component of molecular weight around 45 000, or a dimer of a band 3-com-

ponent, which is less likely as judged from the electrophoretic patterns (Fig. 3).

A small amount of phosphate was found in the active fractions in the experiment of Fig. 4. If this corresponds to phospholipids some of them might be bound to the transport protein, thereby increasing its apparent molecular weight.

The activity of the 3.3-polypeptide (molecular weight 88 000) was estimated assuming that this polypeptide binds cytochalasin B with high affinity ($1.8 \cdot 10^5$ sites/cell [17,21]) and that it transports glucose. The values for equilibrium exchange of glucose in the red blood cell from Ref. 22, used as in Refs. 1 and 11 gives a V for quilibrium exchange of glucose molecules of 1900 site$^{-1} \cdot$ s^{-1}. From the chromatographic data (Fig. 4) and scans of the electrophoretic analysis we have calculated the concentration of the 3.3-polypeptides in the incubation with radioactive glucose to be $3.9 \cdot 10^{10}$ polypeptides per ml, and proceeding as in Refs. 1 and 11 we then calculated an initial uptake of $1.2 \cdot 10^{-7}$ mol glucose per min per ml of incubation mixture, i.e. 300 molecules of glucose per polypeptide per second, which represents an activity recovery (per 3.3-polypeptide or cytochalasin B binding site) of 16%. In the above calculations the initial rate corresponds to 1.3 times the stereospecific uptake per minute measured at two minutes, as estimated from a separate experiment at low concentration of transport protein (not shown). The initial rate probably corresponds to the uptake of glucose through the outermost bilayer of lipids in the multilamellar liposomes. Since this layer constitutes about 40% of the area of the layers, as shown by simple calculations based on electron micrographs, and therefore most probably contains about 40% of the transport protein, the above recovery value of 16% should be multiplied by 100/40. The corrected yield is thus about 40%.

In exactly the same way, for the experiment illustrated in Fig. 3 at a K_{av} value of 0.4, the recovery was 9% X 100/40 or about 22%. Although approximate, these values show that the 3.3-polypeptides can account for the activity. Some of the 3.3-polypeptides might have become inactivated, and some might differ from the glucose transporter.

A comparison between our upper limit for the molecular weight of the stable transporter in cholate, 350 000 ± 20 000, and the apparent molecular weight of the band 3.3-polypeptide, 88 000, indicates that the transporter in cholate might be a tetramer of 3.3-polypeptides, or a dimer in complex with some phospholipids. Even in the former case the functional unit might be a dimer, since the cholate extract might contain complexes of two or more dimers. The molecular weight 185 000 found by electron inactivation experiments by Cuppoletti and Jung for the glucose transporter in the intact membrane [7] supports the hypothesis that the glucose transport is associated with a dimer of 3.3-polypeptides.

The yield upon solubilization reported earlier, 6% [1], can be corrected for the effect of the multilamellar liposomes and for the increased activity in the present extraction conditions to be about 24%, which is very similar to the average yield of 30% for the activity of the band 3.3-polypeptides, which indicates that no loss of activity occurred during the fractionation procedures and strengthens the view that the 3.3-polypeptides can account for the activity. The present interpretations are supported by results obtained by fractionation on a wheat germ lectin column [18].

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