Biochimica et Biophysica Acta, 644 (1981) 101–107
Elsevier/North-Holland Biomedical Press

BBA 79262

THE STEREOSPECIFIC D-GLUCOSE TRANSPORT ACTIVITY OF CHOLATE EXTRACTS FROM HUMAN ERYTHROCYTE MEMBRANES

PER LUNDAHL, FERNANDO ACEVEDO, GUNNAR FRÖMAN and SUREE PHUTRAKUL *

Institute of Biochemistry, Biomedical Center, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)

(Received December 12th, 1980)

Key words: D-Glucose transport; Cholate extract; Phospholipid liposome; Stereospecificity; (Human erythrocyte membrane)

The glucose transport protein of human erythrocyte membranes was solubilized with cholate to facilitate rapid reconstitution and direct glucose transport measurements. This may simplify the isolation of the native glucose transporter. In most experiments the membranes were prepared from fresh blood within 8 h, frozen in liquid nitrogen and stored at −70°C to minimize proteolytic degradation. Solubilization with 25 mM cholate in the presence of 200 mM NaCl at pH 8.4 for 12 min at room temperature gave a high D-glucose transport activity. The solubilized mixture contained 20% of the total membrane protein, only 6% of the polypeptides of molecular weight around 90 000, 23% of the polypeptides of molecular weight around 55 000, 30% of the phospholipids and at least 6% of the stereospecific D-glucose transport activity. At cholate concentrations up to 22 mM the ratio of solubilized phospholipids to cholate increased steeply, concomitant with an increase in solubilized activity. Above 30 mM cholate the activity diminished. At 4°C the activity of the extract decreased rapidly within the first day and slowly during the next few days. The initial changes seem to have produced a fairly stable, but not native form or fragment of the transporter. When 20 mM EDTA and 5 mM dithioerythritol were included in the solubilization mixture a high activity was preserved for about one day.

Introduction

It is still not clear whether the monomer or subunit of the glucose transport protein of human erythrocyte membranes has a molecular weight around 90 000 (band 3 in dodecyl sulfate electrophoresis; denotation as in Ref. 1) or around 55 000 (the 4.5 region). Both alternatives have been repeatedly suggested (cf. Refs. 2–7). Several data derive from indirect evidence utilizing the interaction between the transporter and specific binders, for instance cytochalasin B (see Ref. 4, Table 2), maltosylisothiocyanate [2,3] or antibodies [6,7]. Direct transport measurements after reconstitution of the transport system are mainly limited to experiments in Triton X-100 [7–12] or similar detergents [6]. Some fractionations have been done with reconstituted systems in liposomes [13,14]. The Triton X-100 fractionations have led to the isolation of a component with glucose transport activity that migrates as a diffuse band in the 4.5 region [7–11].

Since the detergent Triton X-100 has a low critical micelle concentration and a big micelle size it cannot be removed from protein solutions as quickly as, for instance, cholate. The micellar properties of cholate make this detergent suitable for experiments in which reconstitution of a transport system is required. As reported earlier, cholate-phospholipid mixtures can solubilize glucose transport activity from human erythrocyte membranes [13,14], but at a high cholate concentration in the absence of exogenous phospholipids the transport is inactivated [14].

We have now studied the solubilization of the glucose transport activity of the human erythrocyte

* Present address as from August 1981: Chemistry Department, Chiang Mai University, Chiang Mai, Thailand.

membrane at low concentrations of cholate, and have found conditions that give a relatively high yield of glucose transport activity. In addition the decrease of activity with time has been determined. We hope that this investigation will facilitate preparation of the native glucose transporter.

Materials and Methods

Membranes. Human erythrocyte membranes were prepared by the molecular sieve method [15] as described earlier [14]. In the first experiments recently outdated blood was used, and the membranes were stored at 4°C in 30 mM NaN_3 (Fig. 1, one experiment in Fig. 2, Fig. 3). In all other cases the membranes were prepared from fresh blood within 8 h and were immediately frozen dropwise in liquid nitrogen. This procedure completely preserved the glucose transport activity. The frozen membranes were stored at −70°C until used.

Chemicals. Cholic acid, egg yolk phospholipids, and radioactive D- and L-glucose were as described in Ref. 14. Sephadex G-50 and prepacked 1.5 × 5 cm Sephadex G-25 columns were from Pharmacia, Sweden.

Solubilization. The membranes were added to a cholate solution and stirred vigorously for 12 min at room temperature (22°C). The composition of the solubilization mixture was normally 25 mM cholate, 200 mM NaCl, 5 mM Tris-HCl, pH 8.4, and 5 mM EDTA, and membranes corresponding to a protein concentration of about 4 g/l and about 4 mM phosphate. The volume was 2–4 ml. The mixture was quickly transferred to centrifuge tubes that were placed in a precooled Spinco 75 Ti rotor and centrifuged at 130 000 × *g* (50 000 rev./min) for 1 h at 2°C. Preliminary molecular sieve chromatography experiments with the extracts in cholate indicate that the non-sedimented material contains no big aggregates of proteins.

Reconstitution of the D-glucose transport system. Three volumes (150 μl) of the solubilized membrane components were mixed with one volume (50 μl) of 260 mM egg yolk phospholipids (200 g/l) in 490 mM cholate, 300 mM NaCl, 22 mM D-glucose, 0.80 mM $MgCl_2$, 0.56 mM $CaCl_2$ and 28 mM 2-mercaptoethanol, pH 8.2. After 3 min at 22°C liposomes were prepared in 5 min by molecular sieve chromatography of 200 μl of sample in a 1.5 × 5 cm (9 ml) Sephadex G-25 column with 10 mM sodium phosphate buffer, pH 7.2, 200 mM NaCl and 5.55 mM D-glucose, at room temperature. The liposomes were collected in a 1.33 ml fraction containing more than 90% of the lipids. That liposome fraction, in an Ellerman tube (10 × 50 mm), was frozen in dry-ice/ethanol (5 min) and thawed in water at 25°C. After 30 min in the water bath the strongly turbid mixture was homogenized with a vortex mixer (3 s) and immediately assayed for glucose transport activity.

Stereospecific D-glucose uptake. Five μl (9 kBq) of ^{14}C-labelled D- or L-glucose were mixed with 200 μl of liposomes. The mixture contained 5.55 mM D-glucose, 200 mM NaCl and 10 mM phosphate buffer, pH 7.2. After 120 s at 22°C 200 μl of 0.20 mM $HgCl_2$ was added along with glucose, NaCl and buffer as above. After 2 min the liposomes were separated from free radioactive glucose on 1.5 × 5 cm Sephadex G-25 columns in the above medium containing 0.10 mM $HgCl_2$. Fractions were collected as described in Ref. 14. The whole procedure was done at room temperature. The stereospecific D-glucose uptake (transport activity) was expressed as D-[^{14}C]-glucose uptake minus L-[^{14}C]glucose uptake, in percent of total radioactivity.

The uptake of L-glucose was in the range of 0.5–3%, and it was about equal to or lower than the stereospecific D-glucose uptake (cf. Ref. 14, Fig. 3). The uptake of L-glucose increased with increasing protein concentration in the reconstitution step.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was done essentially according to the method of Neville [16] as described in Ref. 14. The gels were stained with Coomassie Brilliant Blue R-250 and the absorbance at 583 nm was scanned.

Chemical analyses. Phospholipids were determined by phosphate analyses according to the method of Bartlett [17]. Membrane protein was determined by absorbance measurement in sodium dodecyl sulfate solution [18]. All pH values were determined at room temperature.

Results

Solubilization of the glucose transport activity. To find suitable conditions for a high yield of glucose

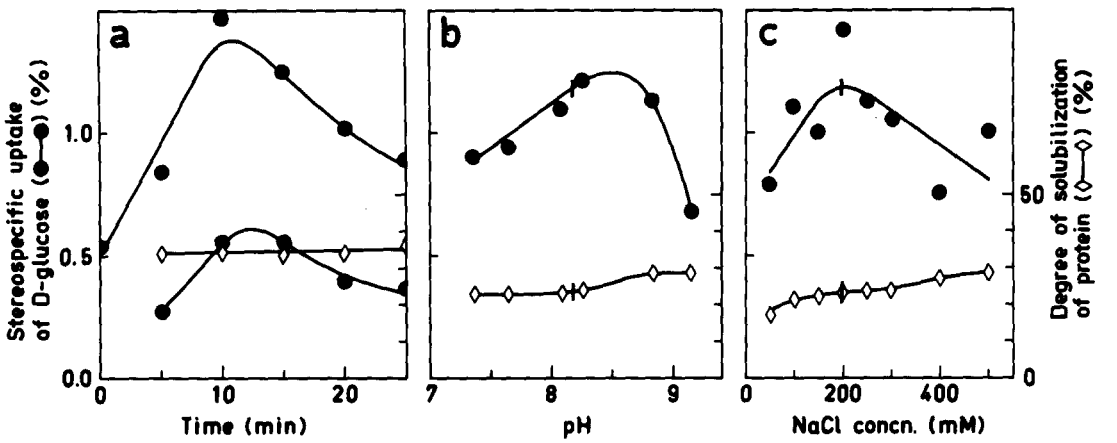

Fig. 1. Effects of time, pH and NaCl concentration on the cholate solubilization of the glucose transport activity and the protein of human erythrocyte membranes. (a) Solubilization as a function of time, at pH 8.2 in 150 mM NaCl. Upper curve: 120 mM cholate, 65 mM egg yolk phospholipids [14], protein concentration 2 g/l. Lower curve: 42 mM cholate, protein concentration 7 g/l. (b) and (c): Solubilization as a function of pH and NaCl concentration at 25 mM cholate, 12 min, protein concentration 4 g/l. (b) 200 mM NaCl, (c) pH 8.2. All the solubilizations were done at room temperature. In the experiment of (b) the pH was adjusted to 8.2 before reconstitution. The stereospecific D-glucose uptake (transport activity) was expressed as D-[^{14}C]glucose uptake minus L-[^{14}C]glucose uptake, in percent of total radioactivity.

transport activity the time, the pH and the concentration of NaCl and of cholate were varied.

Time. Although the activity increased initially (Fig. 1a), longer periods of extraction did not solubilize appreciably more protein. This initial increase was most evident with viscous solutions containing large amounts of phospholipids or of membranes, as in Fig. 1a. The lower curves show a low transport activity and a high solubilization of protein, since the concentration of cholate is above optimal (see Fig. 2). After 15 min the transport protein became inactivated, probably partly by proteolysis. The latter effect was smaller with membranes prepared from fresh blood (not shown).

pH and NaCl. An increase of pH from 7.4 to 9.2 (Fig. 1b) and of the NaCl concentration from 50 to 500 mM (Fig. 1c) caused changes in activity that did not correlate with the overall protein solubilization. At 200 mM NaCl there seems to be a pH optimum at 8.4 (Fig. 1b), and at pH 8.2 the optimal salt concentration is about 200 mM (Fig. 1c). Since pH and ionic strength both affect mainly electrostatic interactions in the ranges tested, their effects are interrelated. Among other effects the concentration of monomeric cholate increases with increasing pH and with decreasing NaCl concentration (cf. Refs. 19 and 20). In a three-dimensional diagram, the curves of Figs. 1b and c would intersect at the points indicated by bars.

Cholate. The solubilization of glucose transport

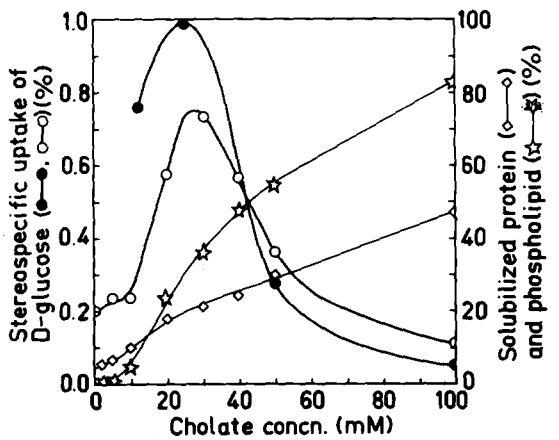

Fig. 2. Effect of cholate concentration on the solubilization of glucose transport activity, protein and phospholipids. Open symbols, protein concentration 4.8 g/l, outdated blood. Filled circles, protein concentration 3.3 g/l, fresh blood, liposomes prepared in a 1.0 X 39 cm Sephadex G-50 column. Each point represents the average value from two experiments.

activity increased with increasing cholate concentration up to about 25 mM, for membranes from fresh as well as from outdated blood (Fig. 2). The amount of extracted protein increased almost linearly with the cholate concentration, whereas the curve for phospholipids was sigmoidal (Fig. 2) with an inflexion point at 22 mM cholate (cf. Fig. 3 and Ref. 21). Up to this point increases in cholate concentration thus have a cooperative effect on the release of phospholipids (Fig. 3).

The solubilization of the glucose transport protein thus seems to increase up to about 20 mM cholate, concomitant with the rapid release of phospholipids, possibly of a special class (but cf. Ref. 21). At higher cholate concentrations the transport protein is inactivated.

The activity at 25 mM cholate increased approximately linearly with increasing protein concentration, and the position of the maximum along the cholate axis moved only slightly toward higher cholate concentration (not shown).

The sialoglycoproteins were solubilized to about 20% as judged from electrophoresis (not shown).

Stability. The glucose transport activity decreased with time, as found by daily measurements of the activity in cholate extracts of fresh membranes from fresh blood. These extracts were prepared with and

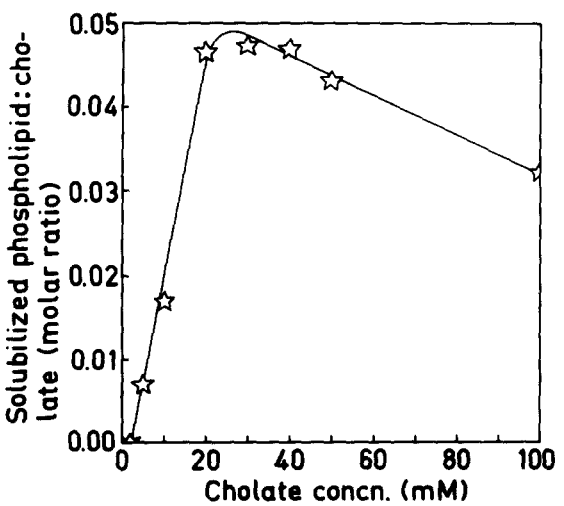

Fig. 3. Illustration of the solubilization of phospholipids. The number of phospholipid molecules solubilized per cholate molecule, as a function of cholate concentration. Data from Fig. 2 (☆).

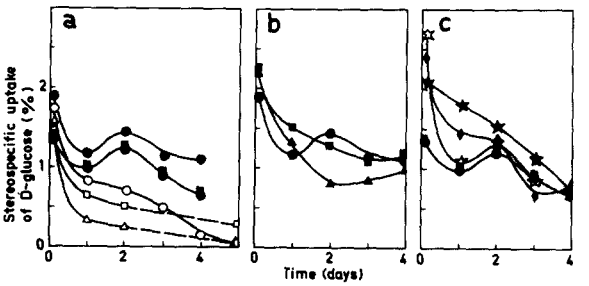

Fig. 4. The cholate-solubilized glucose transport activity during storage at 4°C. Membranes from fresh blood were solubilized at a protein concentration of 4 g/l in the presence of 30 mM NaN_3 and the following compounds: (a–c) ○, control; □, 10 mM 2-mercaptoethanol (ME); △, 0.1 mM phenylmethylsulfonyl fluoride (PMSF); ●, 5 mM EDTA; ♦, 20 mM EDTA; ■, 5 mM EDTA and 10 mM ME; ▲, 5 mM EDTA and 0.1 mM PMSF; ◆, 20 mM EDTA and 10 mM ME; ☆, 20 mM EDTA and 0.5 mM dithioerythritol (DTE); ★, 20 mM EDTA and 5 mM DTE. Liposomes were prepared at the times indicated in the figure. Freeze-thawing and transport measurements were then done immediately. Each point represents the average of duplicate measurements on one batch of liposomes.

without the proteolytic inhibitors EDTA and phenylmethylsulfonyl fluoride, and the reducing agents 2-mercaptoethanol and dithioerythritol.

In the absence of EDTA less than half of the activity remained after one day at 4°C (Fig. 4a). Phenylmethylsulfonyl fluoride, which has been reported to prevent the degradation of the glucose transport protein [3], did not preserve the transport activity in our experiment (Fig. 4a), but it did slow down the proteolysis of at least spectrin (electrophoretic analyses, not shown). 2-Mercaptoethanol alone did not increase the stability.

In the presence of 5 or 20 mM EDTA much more of the activity remained after 1–2 days (Fig. 4a). Phenylmethylsulfonyl fluoride combined with 5 mM EDTA again showed no significant positive effect, and 2-mercaptoethanol increased the activity slightly at days 0 and 1 (Fig. 4b). In 20 mM EDTA with 5 mM dithioerythritol (Fig. 4c) the activity was more stable during the first two days.

Probably the decreases in activity are due to proteolysis, which to some extent can be inhibited by EDTA and perhaps by dithioerythritol, and to oxidation, against which dithioerythritol also protects. Pos-

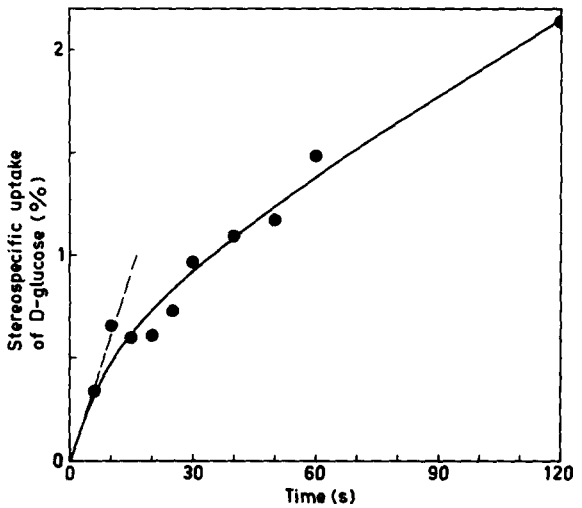

Fig. 5. Time course of stereospecific D-glucose uptake into liposomes. Membranes from fresh blood were solubilized in the presence of 20 mM EDTA and 2 mM dithioerythritol, at a total protein concentration of 3.7 g/l. The liposomes were frozen and thawed and incubated with D-[^{14}C]- or L-[^{14}C]-glucose for various times, as described in Materials and Methods. At the incubation the concentration of protein was 0.081 g/l (corresponding to a concentration of 0.40 g/l of total membrane protein), and the D-glucose concentration was 5.5 mM. The initial slope is indicated by the broken line. The stereospecific uptake was approx. 0.00 at time zero, since the uptake of D-glucose minus the uptake of L-glucose in the presence of 0.10 mM $HgCl_2$ was 0.00–0.04% as determined in a separate experiment. The uncertainty in the baseline of the measurements of the stereospecific uptake was ±0.02 (cf. Ref. 14) as estimated from the elution profiles of D- and L-glucose on the Sephadex columns in the present experiment.

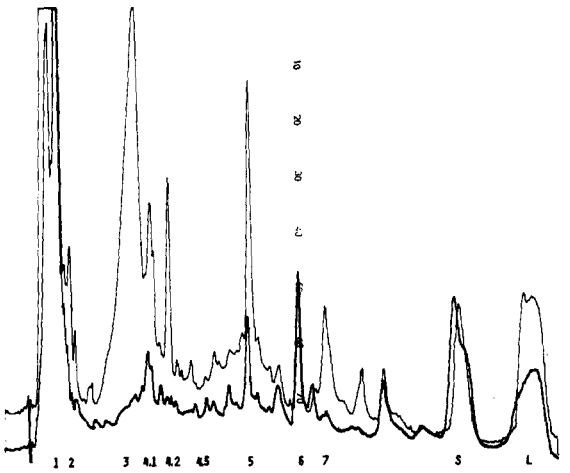

Fig. 6. Electrophoretic patterns of membranes and cholate extract after polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Thin line, 50 μg of total membrane protein from fresh blood. Thick line, 18 μg of solubilized membrane protein from the extract corresponding to the point ☆ (open star) at 0.1 day in Fig. 4c. This amount of protein (18 μg) is solubilized from 88 μg of total membrane protein. When the two curves are compared for estimation of the degree of solubilization of various protein subunits the membrane curve (thin line) should therefore be raised by a factor of 1.8. Note that the baselines do not coincide. The samples were kept at 4°C for less than 1 h before the addition of dodecyl sulfate, and then immediately applied to the gel. Some zones are numbered according to the system of Steck [1]. S, small peptides including the hemoglobin subunit; L, lipids migrating in the electrophoretic front.

sibly the presence of cholate itself causes slow inactivation.

Yield of stereospecific D-glucose transport activity. To estimate the degree of solubilization of the transport activity a rate experiment was done (Fig. 5). The shape of the rate curve is similar to that reported earlier for phospholipid-cholate extracts [14]. The initial slope corresponds to a transport rate of about 0.51 μmol of D-glucose per min and mg of total protein (and five times higher per mg of solubilized protein, since the degree of solubilization is 20%). This gives a yield of 6.5% of the activity in intact red blood cells when estimated exactly as in Ref. 14. This value of 6.5% is a lower limit, since part of the activity might be masked in multilamellar liposomes.

As estimated by polyacrylamide gel electrophoresis of the cholate extract and of membranes (Fig. 6) only 6% of the band 3-protein but 23% of the protein in the 4.5-region is solubilized. The yield of activity is therefore high in relation to the amount of protein that is solubilized, and especially so when the band 3-protein is considered.

Discussion

It has been questioned [2,3,12] whether degradation of a band 3-component could give rise to the active glucose transport component that has been isolated in Triton X-100 [7–11]. However, antibodies against this purified 4.5-component have been shown to react only with the 4.5-region in dodecyl sulfate

acrylamide gels and this has been presented as an argument against a band 3-glucose transporter [6,7]. It is well known that the glucose transport activity in Triton X-100 [11], in cholate-phospholipid [14] and in cholate (this work, Fig. 4) decreases with time, and that proteolytic degradation can take place [2,3,12, 22–27]. Therefore it is desirable to measure glucose transport activity directly and quickly after a simple reconstitution step. This can be done in cholate due to the small micelle size and the relatively high critical micelle concentration [28].

The glucose transporter from human erythrocyte membranes has earlier been solubilized in cholate-lipid mixtures [13,14]. Recently the glucose transport component of rat adipocyte membranes has been solubilized in cholate (49 mM) and fractionated [29]. Cholate at high concentration might denature proteins (cf. Fig. 2), similar to what has been reported for deoxycholate [28]. However, phospholipids, exo- or endogenous, probably lower the concentration of free monomeric or oligomeric cholate. Mixed micelles are formed and act as buffers against changes in the concentration of free cholate (cf. Ref. 19, Table III; Ref. 14, Table I and Figs. 2 and 3 in this work).

The decrease in glucose transport activity with increasing cholate concentration (Fig. 2) might mainly be a direct effect of cholate. In addition, there is a decrease in activity with time (Fig. 4), which can be attributed to proteolysis, oxidation, or denaturation by cholate. This decrease is slowed by the addition of EDTA and dithioerythritol. However, electrophoretic analysis showed that EDTA did not completely inhibit the proteolytic activity. More protein zones appeared in the 4.5-region after two days at 4°C (not shown). Probably there was some degradation of the glucose transport protein.

In our hands the cholate extraction released little of the band 3-protein, the main component of which is known to transport anions [30]. This small amount of solubilized band 3 might constitute the glucose transporter. If that is the case this protein shows a very high specific activity for glucose transport. On the other hand, if the glucose transport protein is a component from the 4.5-region, the activity observed accounts for at least 6/23 or about one fourth of the maximal specific activity.

We suggest that the native glucose transporter can be safely identified if the fractionation can be done in such a short time, or in the presence of such compounds that the activity is preserved throughout the procedures. The necessary activity measurements are considerably simplified by the use of cholate or some other detergent that can be rapidly removed, like octyl glucoside. Fractionation work is in progress in our laboratory.

Acknowledgements

We thank Mrs. Eva Greijer for excellent technical assistance. Valuable suggestions have been made by Dr. Lena Hammar. A grant from The International Seminars, Uppsala, to one of us (S.P.) is gratefully acknowledged. Fresh blood was obtained by the courtesy of the Blood Bank at The Academic Hospital, Uppsala. The work has been financially supported by the O.E. and Edla Johansson Scientific Foundation and by the Swedish Natural Science Research Council.

References

1 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
2 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1199–1205
3 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1205–1212
4 Jones, M.N. and Nickson, J.K. (1980) FEBS Lett. 115, 1–8
5 Jung, C.Y., Hsu, T.L., Hah, J.S., Cha, C. and Haas, M.N. (1980) J. Biol. Chem. 255, 361–364
6 Baldwin, S.A. and Lienhard, G.E. (1980) Biochem. Biophys. Res. Commun. 94, 1401–1408
7 Sogin, D.C. and Hinkle, P.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5725–5729
8 Kasahara, M. and Hinkle, P.C. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 396–400
9 Kahlenberg, A. (1976) J. Biol. Chem. 251, 1582–1590
10 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
11 Kahlenberg, A. and Zala, C.A. (1977) J. Supramol. Struct. 7, 287–300
12 Phutrakul, S. and Jones, M.N. (1979) Biochim. Biophys. Acta 550, 188–200
13 Goldin, S.M. and Rhoden, V. (1978) J. Biol. Chem. 253, 2575–2583
14 Fröman, G., Acevedo, F., Lundahl, P. and Hjertén, S. (1980) Biochim. Biophys. Acta 600, 489–501
15 Fröman, G., Acevedo, F. and Hjertén, S. (1980) Prep. Biochem., 10, 59–67
16 Neville, D.M., Jr. (1971) J. Biol. Chem. 246, 6328–6334

17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
18 Lundahl, P. (1975) Biochim. Biophys. Acta 379, 304–316
19 Carey, M.C. and Small, D.M. (1970) Am. J. Med. 49, 590–608
20 Carey, M.C. and Small, D.M. (1972) Arch. Intern. Med. 130, 506–527
21 Coleman, R., Holdsworth, G. and Finean, J.B. (1976) Biochim. Biophys. Acta 436, 38–44
22 Bernacki, R.J. and Bosmann, H.B. (1972) J. Membr. Biol. 7, 1–14
23 Tökés, Z.A. and Chambers, S.M. (1975) Biochim. Biophys. Acta 389, 325–338
24 Brovelli, A., Suhail, M., Pallavicini, G., Sinigaglia, F. and Balduini, C. (1977) Biochem. J. 164, 469–472
25 Murakami, T., Suzuki, Y. and Murachi, T. (1979) Eur. J. Biochem. 96, 221–227
26 Pontremoli, S., Salamino, F., Sparatore, B., Melloni, E., Morelli, A., Benatti, U. and De Flora, A. (1979) Biochem. J. 181, 559–568
27 Tarone, G., Hamasaki, N., Fukuda, M. and Marchesi, V.T. (1979) J. Membr. Biol. 48, 1–12
28 Helenius, A., McCaslin, D.R., Fries, E. and Tanford, C. (1979) Methods Enzymol. 56, 734–749
29 Carter-Su, C., Pillion, D.J. and Czech, M.P. (1980) Biochemistry 19, 2374–2385
30 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302