Cogdell, R. J., & Thornber, J. P. (1980) *FEBS Lett. 122*, 1–8.
Duysens, L. N. M. (1956) *Biochim. Biophys. Acta 19*, 1–12.
Feher, G., & Okamura, M. Y. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 349–386, Plenum Press, New York.
Heathcote, P., Vermeglio, A., & Clayton, R. K. (1977) *Biochim. Biophys. Acta 461*, 358–365.
Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28–32.
Huang, C. (1969) *Biochemistry 8*, 344–351.
Hwang, S. B., & Stoeckenius, W. (1977) *J. Membr. Biol. 33*, 325–350.
Jacrot, B. (1976) *Rep. Prog. Phys. 39*, 911–953.
Jacrot, B., & Zaccai, G. (1981) *Biopolymers 20*, 2413–2426.
Jolchine, G., & Reiss-Husson, F. (1974) *FEBS Lett. 40*, 5–8.
Knoll, W., Haas, J., Stuhrmann, H. B., Fuldner, H. H., Vogel, H., & Sackman, E. (1981a) *J. Appl. Crystallogr. 14*, 191–202.
Knoll, W., Ibel, K., & Sackmann, E. (1981b) *Biochemistry 20*, 6383–6397.
Lowry, O. H., Rosenbrough, W. J., Farr, A. L., & Rendall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 71–123, Academic Press, New York.
Nabedryk, E., & Breton, J. (1981) *Biochim. Biophys. Acta 635*, 515–524.
Nabedryk, E., Tiede, D. M., Dutton, P. L., & Breton, J. (1982) *Biochim. Biophys. Acta 682*, 273–280.
Overfield, R. E., & Wraight, C. A. (1980a) *Biochemistry 19*, 3322–3327.
Overfield, R. E., & Wraight, C. A. (1980b) *Biochemistry 19*, 3328–3334.
Pachence, J. M., Dutton, P. L., & Blasie, J. K. (1979) *Biochim. Biophys. Acta 548*, 348–373.
Pachence, J. M., Dutton, P. L., & Blasie, J. K. (1981) *Biochim. Biophys. Acta 635*, 267–283.
Pachence, J. M., Dutton, P. L., & Blasie, J. K. (1983) *Biochim. Biophys. Acta 724*, 6–19.
Pearson, R. H., & Pascher, I. (1979) *Nature (London) 281*, 499–501.
Reinsch, C. (1961) *Z. Phys. 163*, 424–434.
Reiss-Husson, F. (1967) *J. Mol. Biol. 25*, 363–382.
Reiss-Husson, F., De Klerk, H., Jolchine, G., Jauneau, E., & Kamen, M. D. K. (1971) *Biochim. Biophys. Acta 234*, 73–82.
Rhoden, V., & Goldin, S. M. (1979) *Biochemistry 18*, 4173–4176.
Rivas, E., Reiss-Husson, F., & Le Maire, M. (1980) *Biochemistry 19*, 2943–2950.
Rothschild, K. J., & Clark, N. A. (1979) *Biophys. J. 25*, 473–487.
Sadler, D. M., & Worcester, D. L. (1982) *J. Mol. Biol. 159*, 485–499.
Schmatz, W., Springer, T., Schelten, J., & Ibel, K. (1974) *J. Appl. Crystallogr. 7*, 96–116.
Sears, V. F. (1975) *Adv. Phys. 24*, 1–45.
Singleton, W. S., Gray, M. S., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
Stuhrmann, H. B. (1974) *J. Appl. Crystallogr. 7*, 173–178.
Stuhrmann, H. B., & Duce, E. D. (1975) *J. Appl. Crystallogr. 8*, 583–542.
Szoka, F. C., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.
Valkirs, G. E., & Feher, G. (1982) *J. Cell Biol. 95*, 179–188.

A Rapid Method of Reconstituting Human Erythrocyte Sugar Transport Proteins†

A. Carruthers* and D. L. Melchior*

ABSTRACT: A rapid reconstitution procedure for human erythrocyte hexose transfer activity is described. The procedure (reverse-phase evaporation) avoids exposure of the isolated proteins to detergent, organic solvent, sonication, or freeze–thaw steps during insertion into synthetic membranes and may be effected within 15 min. The so-formed vesicles are unilamellar structures with a large encapsulated volume, narrow size range, and low passive permeabilities. Contamination by carry-through of endogenous (red cell) lipids is less than 1%. Reconstituted hexose transfer activity was examined by using unfractionated proteins (bands 3, 4.5, and 6) and purified proteins (bands 4.5 and 3). With unfractionated proteins, hexose transport activity is low [0.34 μmol·(mg of protein)$^{-1}$·min^{-1}], is inhibited by cytochalasin B, and increases monotonically with protein concentration. Kinetic analysis indicates that V_{max} values for both influx and efflux of D-glucose are identical. Reconstitution of the cytochalasin B binding protein (band 4.5) results in hexose transport with high specific activity [5 μmol·(mg of protein)$^{-1}$·min^{-1}] and symmetry in transfer kinetics. Band 3 proteins also appear to mediate cytochalasin B sensitive D-glucose transport activity.

The sugar transport proteins of the human erythrocyte membrane have been reconstituted into synthetic membranes by a variety of means. These methods include reconstitution by detergent dialysis (Goldin & Rhoden, 1978), reconstitution by insertion into planar black lipid membranes (Jones & Nickson, 1978), or reconstitution by a freeze–thaw, sonication procedure (Kasahara & Hinkle, 1977; Wheeler & Hinkle, 1981). Here we report a rapid reconstitution procedure that obviates the need for exposing proteins to detergent, organic solvent, sonication, or freeze–thaw cycles during insertion into synthetic membranes (reverse-phase evaporation; Szoka & Paphadjopoulos, 1980; Düzgünes et al., 1983). The vesicles formed by this procedure are large unilamellar structures of a narrow size range, with hexose transfer characteristics similar

† From the Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01605. *Received January 11, 1984.* This work was funded in part by NIH Grant AM-32520. A.C. gratefully acknowledges assistance from the MDA and a travel award from the Wellcome Foundation. A.C. is a NATO SERC (Great Britain) Overseas Fellow.

0006-2960/84/0423-2712$01.50/0

to those of vesicles produced by other methods (Kasahara & Hinkle, 1977; Goldin & Rhoden, 1978; Baldwin et al., 1981; Shelton & Langdon, 1983). Our findings support the views that more than one protein type may mediate native transport (Goldin & Rhoden, 1978; Jones & Nickson, 1981; Shelton & Langdon, 1983) and that more than one reconstitution procedure may be necessary to demonstrate this.

Materials and Methods

Preparation of Red Cell Ghosts. Human erythrocyte ghosts were prepared from outdated blood bank whole blood as described previously (Carruthers & Melchior, 1983a).

Membrane Protein Solubilization and Purification. Red cell ghosts were incubated in 25 mM NaCl, 5 mM Tris-HCl,[1] 0.2 mM EDTA, and 0.5% Triton X-100, pH 8, for 30 min at 4 °C (1 mL of ghosts per 5 mL of buffer). The resulting suspension was centrifuged at 45000*g* for 30 min and the supernatant collected and stored on ice. This initial purification procedure solubilizes red cell membrane protein bands 3, 4.5, and 6 (50% of total membrane protein) and 95% of the lipid (Yu et al., 1973; Maddy, 1982). The pellet formed from the remaining proteins (bands 1, 2, 4.1, 4.2, 5, and 7 and 25% of total bands 3, 4.5, and 6 proteins), and lipid was washed once more with the same solution followed by centrifugation. The combined supernatants were concentrated by ultrafiltration (Diaflo YM10 membranes) and stored at −70 °C. This protein solution is the unfractionated membrane protein fraction described under Results.

Band 4.5 proteins were separated from band 3 and 6 proteins by application of the membrane extract to a DEAE-cellulose column (20 × 1.8 cm) equilibrated with 0.5% Triton X-100 in 25 mM NaCl, 5 mM Tris-HCl, and 0.2 mM EDTA, pH 8. The flow-through fraction contains band 4.5 protein, residual hemoglobin, and lipid/Triton X-100 mixed micelles (Kasahara & Hinkle, 1977). Band 3 and 6 proteins were eluted with 1 M NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 8, and 0.5% Triton X-100 (Kasahara & Hinkle, 1977). Band 4.5 and 3 fractions were further concentrated by ultrafiltration. The band 3 fraction was dialyzed against 100 volumes of 25 mM NaCl solution (see above) to reduce the NaCl concentration (4 °C; 14 h). Both fractions were stored at −70 °C.

Reconstitution of Hexose Transfer Activity. Triton X-100 was removed from the protein fractions by addition of Bio-Beads SM-2, followed by gentle agitation at 4 °C for 4–14 h [0.6 g of beads/2 mL of protein solution (10–1000 μg of protein/mL); Holloway, 1973]. This produces a suspension of small unilamellar vesicles formed from residual lipid and protein. The Triton X-100 content of this suspension was reduced by more than 99.8%. The protein content of this nominally Triton X-100 free extract was reduced by 26%. This percentage seemed to be independent of the starting protein concentration. The Triton X-100 free protein suspension was used immediately for reconstitution into synthetic membranes.

Reconstitution was effected by two different procedures—reverse phase evaporation (Szoka & Papahadjopoulos, 1980; Carruthers & Melchior, 1983b) and the method of Kasahara & Hinkle (1977) as modified by Wheeler & Hinkle (1981). With both methods, 25 mg of phospholipid in 0.25 mL of hexane was dried down to a thin film on the bottom of a 50-mL round-bottom flask under N_2. The remaining organic solvent was removed in vacuo overnight. Reconstitution by the method of Wheeler & Hinkle (1981) then proceeded unmodified.

With the reverse-phase evaporation procedure, 1.5 mL of diethyl ether was added to the lipids, followed by 0.25 mL of buffer (25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.5). This mixture was sonicated in a bath sonicator for 2 min, forming a visually homogeneous suspension. The ether was removed completely by rotary evaporation, resulting in a lipidic gel. Large unilamellar vesicles may then be formed by dispersion of the gel in buffer (Szoka & Paphadjopoulos, 1980; Carruthers & Melchior, 1983b). Membrane protein reconstitution was effected by inclusion of the protein in the buffer (25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, pH 7.5) used to disperse the gel. The size of these large unilamellar vesicles was determined, by negative-stain electron microscopy, to be 0.12 ± 0.02 μm. This size range is close to that of vesicles formed by the method of Kasahara & Hinkle (1977). This vesicle size is, however, somewhat lower than that of vesicles formed by reverse-phase evaporation in the absence of protein (0.2 μm; Düzgünes et al., 1983). The calculated Triton X-100:lipid molar ratio of vesicles formed by both procedures was always less than 1:1200. Vesicles formed by both procedures were packed by centrifugation at 20000*g* for 15 min.

Hexose Transfer Determinations. D-Glucose uptake and efflux in the reconstituted systems were measured either by use of radiolabeled D-glucose or by the turbidimetric method (Lefevre, 1948; Sen & Widdas, 1962; Masaik & LeFevre, 1977; Carruthers & Melchior, 1983a,b). Sugar efflux was measured under zero-trans conditions (external sugar absent) and influx under infinite-cis conditions (external concentration of sugar saturating). The initial rate of D-glucose flux was obtained by regression analysis of the initially linear segment of the time course data.

With radiolabeled sugar efflux experiments, vesicles were loaded with 30 mM D-[U-^{14}C]glucose by incubation at 4 °C for 12 h. At this time the calculated intravesicular glucose concentration is 33.4 ± 0.2 mM ($D\text{-}Glu_i/D\text{-}Glu_0 = 1.1 \pm 0.01$). Washed, packed vesicles (5 μL) were placed into 2-mL plastic tubes by using a 20-μL glass Hamilton syringe, and the efflux experiment was initiated by addition of 1 mL of isotope-free, sugar-free buffer. After the required efflux interval, fluxes were arrested by addition of 0.5 mL of ice-cold buffer containing 1.5 mM $HgCl_2$ [see Wheeler & Hinkle (1981)]. The tubes were centrifuged (2 min, Eppendorf bench centrifuge, 5414) and the pellets washed in 1.5 mL of ice-cold buffer containing 500 μM $HgCl_2$. The tip of the plastic tube was removed and counted. Control experiments indicated that this procedure reduces the extravesicular activity by more than 99%. Influx of labeled D-glucose was measured by filtration. Packed vesicles (2 μL) were placed on Millipore filters (0.2-μm pore) by using a preset 20-μL glass syringe, and flux was initiated by addition of a 50-μL solution containing radiolabel. After 15 s the flux was arrested by rapid mixing with ice-cold buffer containing 1 mM $HgCl_2$ followed by rapid filtration. Filters were then collected and counted.

Glucose transport measurements using the turbidimetric method are based on the osmotic behavior of the vesicles. The vesicles have a water permeability some 1000–10 000-fold greater than protein-mediated glucose permeability and behave as perfect osmometers (Carruthers & Melchior, 1983b). Vesicles swell when D-glucose penetrates the intravesicular space and shrink when glucose is lost from the vesicles. As the vesicles act as osmometers, their size is related directly to intravesicular glucose concentration. The time course of sugar transport induced vesicle size change may be monitored conveniently by measuring the turbidity of the suspension of

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; LUVs, large unilamellar vesicles.

vesicles, as turbidity is inversely proportional to vesicle-encapsulated volume (Bangham et al., 1965; Carruthers & Melchior, 1983b). Turbidity was measured on the computer-controlled digital Micro-Turbidimetric Analysis stop-flow system described by Carruthers & Melchior (1983a,b). The reconstituted system (0.5–1 μL of packed vesicles), formed in NaCl buffer, was injected into 400 μL of NaCl buffer containing 30–50 mM D-glucose and the time course of turbidity change monitored. Efflux of glucose was monitored by injecting vesicles loaded with 30 mM glucose into glucose-free NaCl buffer.

Analytical Procedures. Triton X-100 assays were performed according to the method of Garewal (1973) with modification by Lukacovic et al. (1981). Protein assays were as described by Lowry et al. (1951). Triton X-100 interference with the assay was circumvented by inclusion of 3% sodium dodecyl sulfate into the alkaline copper reagent (Yu & Steck, 1975). Polyacrylamide gel electrophoresis was carried out on 7% gels as described by Laemmli (1970). Phospholipid phosphorus assays were as described by Bartlett (1959). Lipids were extracted from LUVs by the method of Bligh & Dyer (1959). Fatty acid analysis was carried out by forming the fatty acid methyl esters in a 14% methanolic BF_3 solution. Samples were run on a Varian 3700 gas chromatograph (Palo Alto, CA) with a 30-m SP2330 capillary column (Supelco, Bellfonte, PA); peaks were detected with a flame ionization detector and integrated on a Hewlett-Packard 1090 reporter–integrator (Avondale, PA).

Determination of Kinetic Constants. Initial rate/concentration data were analyzed according to the method of Wilkinson (1961). K_m and V_{max} for net infinite-cis uptake and net zero-trans exit were obtained by integration of the time course of flux (Hankin et al., 1972; Carruthers & Melchior, 1983a).

Passive Permeability Properties of Vesicles. The passive fluxes of water and D-glucose across the liposomal membrane were estimated as described by Carruthers & Melchior (1983b). Estimates of encapsulated, vesicle volume were made by determination of total and extravesicular water content of pelleted vesicles using [^{3}H]inulin and wet and dry weight analysis (Carruthers & Melchior, 1983b).

Cytochalasin B Binding. The concentration dependence of cytochalasin B binding to reverse-phase LUVs was determined by incubation of pelleted LUVs (10 μL) in 100 μL of [^{3}H]-cytochalasin B–NaCl buffer at 23 °C for 2 h. Following incubation, the LUVs were pelleted by centrifugation at 90000*g* for 20 min, the supernatant was aspirated, and the pellet was dissolved in 5% Triton X-100 and counted.

Contamination by extravesicular activity was 4.6%. D-Glucose-sensitive [^{3}H]cytochalasin B binding was determined by incubation in the presence or absence of 500 mM D-glucose. The apparent half-time for binding equilibrium was 12 min.

Results

Characterization of the Liposomes. The average diameter of the reconstituted egg lecithin vesicles formed by reverse-phase evaporation (Szoka & Paphadjopoulos, 1980) was determined by negative-stain electron microscopy to be 0.12 ± 0.02 μm. Both this procedure and phase-contrast light microscopy show the vesicles to be unilamellar in structure. This is further supported by estimates of an encapsulated volume of 3.12 ± 0.06 μL/μmol of lipid (n = 6). Assuming the vesicles are spherical and the average area of a lipid molecule is 50 Å^2 (Bangham et al., 1965), we can calculate that each vesicle should have an encapsulated volume of 3 μL/μmol of lipid. If an additional lamella of half the diameter of the liposome were present within the structure, the encapsulated volume would be 2.4 μL/μmol of lipid. The passive permeabilities of these vesicles to water and D-glucose (measured in the presence of the sugar transport inhibitor cytochalasin B, 50 μM) are 6×10^{-3} and 1.1×10^{-9} cm/s, respectively (temperature, 20 °C; six separate determinations in each case). These values are in close agreement with earlier estimates in both protein-free and protein-containing synthetic membranes (Bangham et al., 1965; Carruthers & Melchior, 1983b) and attest to the structural integrity of the liposomes. The contamination of reverse-phase LUVs by carry-through of red cell lipid during reconstitution was assessed by fatty acid analysis of extracted dimyristoyllecithin (DML) LUVs containing or lacking band 4.5 proteins (200 μg of protein/40 mg of DML). The small unilamellar vesicles formed by Triton X-100 removal from band 4.5 proteins contain a variety of fatty acids ranging from C14 to C20. These constitute 0.76% of the total fatty acid content of DML LUVs containing band 4.5 proteins.

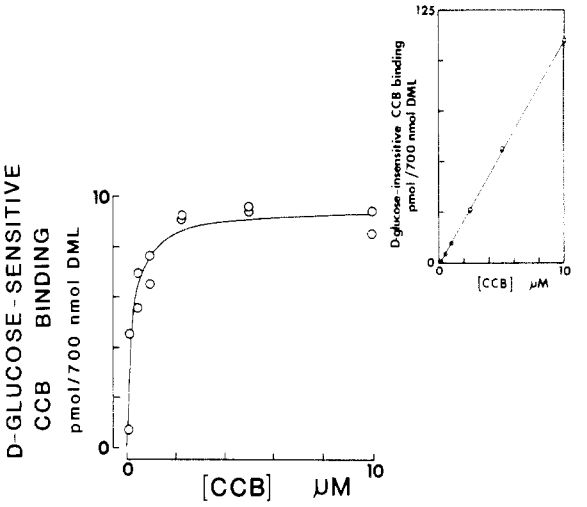

FIGURE 1: Concentration dependence of [^{3}H]cytochalasin B binding to dimyristoyllecithin (DML) LUVs containing band 4.5 protein fraction. Ordinate: D-Glucose (500 mM) displaceable cytochalasin B binding (pmol/700 nmol of phospholipid phosphorus). Abscissa: Cytochalasin B concentration (μM). The LUVs were formed by reverse-phase evaporation using a protein fraction 4.5:lipid ratio (by weight) of 0.2:40. The curve drawn through the points was obtained by nonlinear regression using the procedure of Wilkinson (1961). The derived constants are K_m = 0.37 ± 0.13 μM and maximum binding = 10.14 ± 0.96 pmol/700 nmol of DML. The inset shows the magnitude of nonspecific (D-glucose-insensitive) [^{3}H]cytochalasin B binding; temperature, 22 °C. Each point is the mean of duplicate samples.

Figure 1 shows the concentration dependence of [^{3}H]cytochalasin B binding to these DML LUVs containing band 4.5 proteins. Apparent binding consists of two components—a large, nonspecific component that persists in the presence of 500 mM D-glucose and a smaller, saturable component abolished by 500 mM D-glucose. The nonspecific component is in part (9%) accounted for by equilibration of label with the intravesicular space. Assuming the remainder of the nonspecific binding is associated with lipid, we calculate a molar ratio for binding of cytochalasin B to lipid of 1:7000. Half-maximum binding of cytochalasin B to D-glucose-sensitive sites occurs at 0.37 ± 0.13 μM cytochalasin B with maximum binding of 10.14 ± 0.96 pmol/700 nmol of lipid. Assuming an average molecular weight for band 4.5 protein of 55 000, the ratio of reconstituted protein to phospholipid is 70 μg/40 mg, which is close to the starting value of 200 μg/40 mg. The number of band 4.5 proteins per vesicle is approximately 5.

D-Glucose Fluxes in Egg Lecithin Liposomes Containing Unfractionated Proteins. D-Glucose uptake and efflux in the

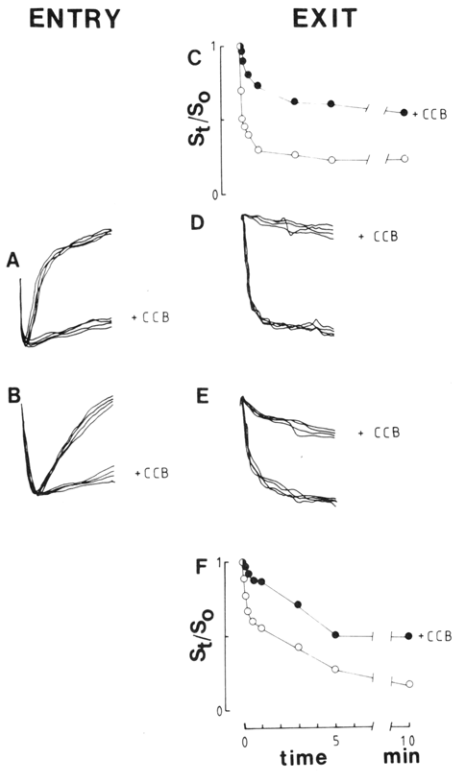

FIGURE 2: D-Glucose entry and exit in the reconstituted system formed from unfractionated proteins by reverse-phase evaporation (B, E, F) or by the method of Kasahara and Hinkle (A, C, D). Uptake was monitored by turbidimetry (A, B) and exit by both turbidimetry (D, E) and use of ^{14}C-labeled D-glucose (C, F). A number of turbidity records are shown superimposed. Radiolabeled D-glucose fluxes were made in triplicate. All fluxes were monitored in the presence and absence of 50 μM cytochalasin B (CCB). Initial intravesicular D-glucose concentration in exit experiments was 30 mM. Uptake of D-glucose was measured from buffer containing 30 mM sugar. The reconstituted systems were each formed from 25 mg of egg phosphatidylcholine and 3.8 mg of protein. The temperature was 30.6 ± 0.2 °C.

reconstituted systems formed both by reverse-phase evaporation and by the procedure of Wheeler & Hinkle (1981) are mediated by two functional pathways: cytochalasin B sensitive and insensitive routes (Figure 2). These pathways correspond to the saturable and nonsaturable transport systems described previously (Goldin & Rhoden, 1978; also see below).

Figure 3 shows the concentration dependence of the inhibition of the initial rate of D-glucose uptake by cytochalasin B in the reverse-phase reconstituted system. Uptake of glucose from solution containing 30 mM sugar was inhibited by 50% at 0.52 ± 0.07 μM cytochalasin B. Moreover, the inhibition of uptake becomes saturated at higher cytochalasin B levels.

Cytochalasin B inhibitable hexose transfer activity increases with increasing protein concentration. This was determined by varying the protein:lipid ratio (by weight) between 1:66 and 1:6.6 during the reconstitution step. Over this range, D-glucose uptake increases monotonically with protein concentration. The cytochalasin B inhibitable hexose transport activity of these LUVs is 0.34 ± 0.05 μmol·(mg of protein)$^{-1}$·min^{-1}. The apparent K_i for inhibition of uptake by cytochalasin B was not affected by increasing protein concentration.

D-Glucose Fluxes in Egg Lecithin Liposomes Containing Fractionated Proteins. Band 4.5 proteins and band 3 proteins (band 3 contained a minor band 6 component, 10%) were reconstituted into bilayers by the method of reverse-phase evaporation and that of Wheeler & Hinkle (1981). Sugar efflux was monitored either by radiolabeled D-glucose or by turbidimetry. Figure 4 illustrates our findings. Band 4.5

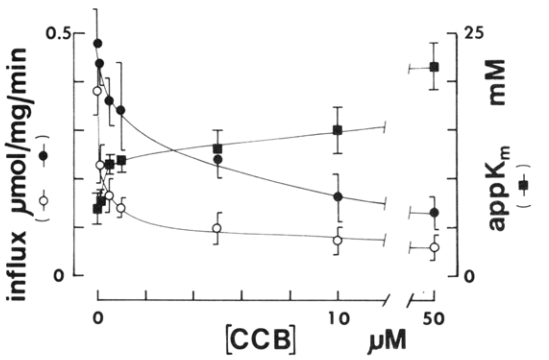

FIGURE 3: Inhibition of D-glucose uptake by cytochalasin B. The reconstituted system was formed from unfractionated proteins and egg phosphatidylcholine (3.8 mg of protein, 25 mg of lipid). Ordinate (○, ●): Uptake [μmol·(mg of protein)$^{-1}$·min^{-1}]. Abscissa: Cytochalasin B concentration (μM). Ordinate (■): K_m^{app} for infinite-cis uptake (mM). The effects of cytochalasin B on the initial rate of uptake from 30 mM D-glucose solution were monitored by turbidimetry and are shown by the open circles (○). Integration of the time course data (see Figure 5) provides estimates of K_m (■) and V_{max} (●). Each point represents the mean ± 1 SD of at least six separate measurements. The temperature is 30.6 ± 0.2 °C. The smooth curves are drawn by eye.

proteins mediate cytochalasin B sensitive D-glucose fluxes when inserted into bilayers by either reconstitution technique. In keeping with earlier findings (Kasahara & Hinkle, 1977; Jones & Nickson, 1981), band 3 proteins are incapable of mediating cytochalasin B inhibitable sugar fluxes when inserted in bilayers with the procedure of Kasahara & Hinkle (1977). However, the same protein fraction confers cytochalasin B sensitive glucose transport activity to vesicles formed by reverse-phase evaporation. The observation is independent of the means of sugar flux determination (Figure 4) and was reproduced consistently in seven separate experiments. The reconstitution procedure of Wheeler & Hinkle (1981) exposes the proteins to freeze–thaw and sonication steps, which are absent in the reverse-phase reconstitution procedure. These steps seem not to account for the disparate behavior of the reconstituted systems. Sonication or rapid freezing followed by thawing of band 3 proteins prior to reconstitution by reverse-phase evaporation is without effect on the transport capacity of the vesicles [control cytochalasin B sensitive initial influx at 30 mM external D-glucose = 0.43 ± 0.05 μmol·(mg of protein)$^{-1}$·min^{-1}, n = 15; proteins quick-frozen and then thawed prior to reconstitution, flux = 0.43 ± 0.06 μmol·(mg of protein)$^{-1}$·min^{-1}, n = 12; proteins sonicated in a bath sonicator for 20 s prior to reconstitution, flux = 0.46 ± 0.13 μmol·(mg of protein)$^{-1}$·min^{-1}, n = 14; temperature, 30.6 ± 0.2 °C].

Figure 5 shows the concentration dependence of the initial rate of D-[^3H]glucose uptake in vesicles containing band 4.5 protein reconstituted by reverse-phase evaporation. Uptake saturates at higher glucose concentrations. The relationship between flux and sugar concentration is well approximated by Michaelis–Menten kinetics with a K_m^{app} of 8.4 ± 3.0 mM and V_{max} of 5.0 ± 0.6 μmol·(mg of protein)$^{-1}$·min^{-1}. This demonstration of saturation kinetics permits us to evaluate K_m and V_{max} values for uptake or efflux by the method of integration of time course data (Hankin et al., 1972). Figure 5 shows such experiments with infinite-cis uptake (50 mM external D-glucose, system 86% saturated). The obtained kinetic parameters are K_m^{app} = 7.5 ● 1.6 mM and V_{max} = 6.0 ± 1.1 μmol·(mg of protein)$^{-1}$·min^{-1} (n = 18). This estimated Michaelis constant for reconstituted hexose transfer is in excellent agreement with measurements of the K_m^{app} for D-glucose

Table I: Kinetics of D-Glucose Transport in LUVs Formed by Reverse-Phase Evaporation

	experiment [a]					
	zero-trans exit			infinite-cis uptake		
	K_m [b]	V_{max} [c]	n [d]	K_m [b]	V_{max} [c]	n [d]
unfractionated proteins	7.6 ± 0.9	0.52 ± 0.07	17	6.8 ± 1.2	0.47 ± 0.08	14
band 4.5 proteins	8.4 ± 1.1	6.5 ± 0.9	15	7.5 ± 1.6	6.0 ± 1.1	18

[a] Zero-trans exit experiments were performed on LUVs loaded with 30 mM D-glucose. Infinite-cis uptake experiments were carried out in NaCl medium containing either 30 mM D-glucose (unfractionated proteins) or 50 mM D-glucose (band 4.5 proteins). Michaelis–Menten constants were obtained by integration of the time course of sugar flux monitored with turbidimetry. [b] mM ± SE. [c] μmol·(mg of protein)$^{-1}$·min^{-1} ± SE. [d] n indicates the number of experiments. Temperature, 29.4 ± 0.8 °C.

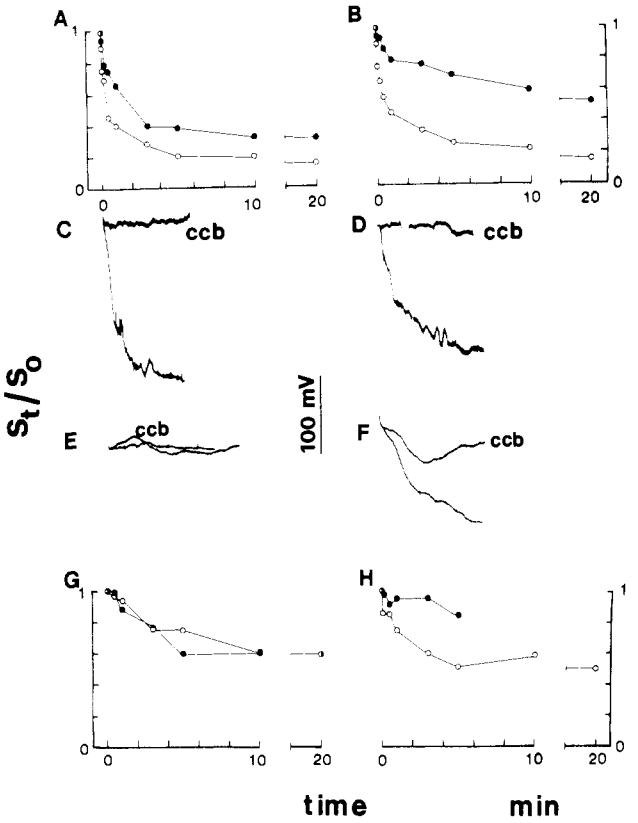

FIGURE 4: D-Glucose effluxes in the reconstituted systems formed by either reverse-phase evaporation (B, D, F, H) or the method of Kasahara and Hinkle (A, C, E, G). Both protein band 4.5 mediated effluxes (A, B, C, D) and band 3 mediated effluxes (E, F, G, H) were monitored by either labeled D-glucose (A, B, G, H) or turbidimetry (C, D, E, F). Fluxes were measured in the presence (●) and absence (○) of 50 μM cytochalasin B (ccb). The D-glucose content of the vesicles prior to efflux was 30 mM. Vesicles were formed from 25 mg of egg phosphatidylcholine and either 0.1 mg of band 4.5 protein or 3.5 mg of band 3 protein. The temperature was 30.6 ± 0.2 °C.

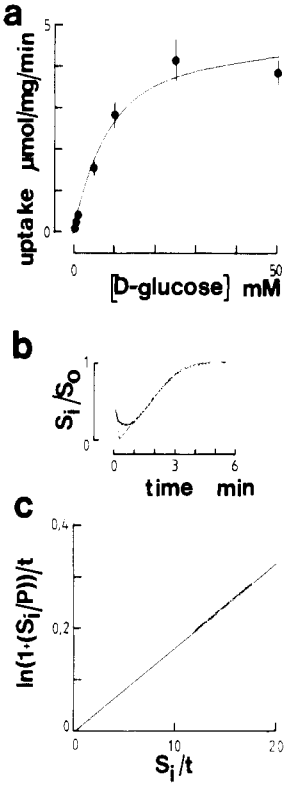

FIGURE 5: Kinetics of D-glucose uptake by the reverse-phase evaporation reconstituted system containing band 4.5 proteins (25 mg of egg phosphatidylcholine/0.1 mg of band 4.5 protein). (a) Concentration dependence of the initial rate of D-[U-^{14}C]glucose uptake. Ordinate: Uptake [μmol·(mg of protein)$^{-1}$·min^{-1}]. Abscissa: External D-glucose concentration (mM). Uptake is shown as that portion of uptake inhibited by 50 μM cytochalasin B. Each point represents the mean ± 1 SE of three separate paired estimates. The smooth curve drawn through the points corresponds to a section of a rectangular hyperbola with K_m^{app} = 8.4 mM and V_{max} = 5 μmol·(mg of protein)$^{-1}$·min^{-1}. The temperature was 27.4 °C. (b) Turbidimetric analysis of the time course of D-glucose uptake from a solution containing 50 mM D-glucose. Eighteen records were digitized and normalized (maximum absorption change was normalized). Ordinate: Estimated fractional equilibration with extravesicular D-glucose. Abscissa: Time (min). Initial absorption was estimated by extrapolation of shrinkage and swelling curves to a common point (Bangham et al., 1965; Carruthers & Melchior, 1983b). The temperature was 28.6 ± 0.2 °C. (c) Estimation of K_m and V_{max} for infinite-cis uptake by transformation of the data from (b) as suggested by Hankin et al. (1972). Such a plot has a slope of $(K_m + P + S_p)/[P(P + S_o)]$ = 0.01639 and y intercept of $-V_{max}K/[P(P + S_o)]$ = −0.00763, where P is the osmolality of glucose-free intra- and extravesicular buffers (65 mOsm) and S_o is the external D-glucose concentration (50 mM). K_m and V_{max} are therefore 7.5 mM and 7.6 mmol·L^{-1}·min^{-1}, respectively.

transport in human erythrocyte white ghosts and inside-out vesicles (7–12 mM; Carruthers & Melchior, 1983a). In view of the close correspondence between initial rate and integration data, we reevaluated the effects of cytochalasin B on net infinite-cis uptake by integration of our data records. The results of this analysis are shown in Figure 3. Cytochalasin B increases the K_m^{app} and reduces V_{max} for infinite-cis D-glucose uptake. The zero-trans net exit data of Figures 2 and 4 may also be analyzed by integration. Table I summarizes these results.

Discussion

Our initial goal was to develop a rapid reconstitution procedure for human erythrocyte hexose transfer that produced large unilamellar vesicles of a narrow size range without the use of detergent. Such a reconstituted system would be more accessible to experimental manipulation than those produced by detergent dialysis (Goldin & Rhoden, 1978). Moreover, a narrow size range and large vesicle size would permit the accurate kinetic analysis of reconstituted transport. The procedure we adopted was reconstitution by reverse-phase evaporation (Szoka & Papahadjopoulos, 1980). Vesicles

formed in this way are unilamellar with a large encapsulated volume and hence are better suited to transport studies than smaller vesicles such as those produced by dialysis. Reconstitution can be effected within 15 min, which is many times more rapid than reconstitution by detergent dialysis (Goldin & Rhoden, 1978).

The features of transport reconstituted by this procedure were examined either by using radiolabeled D-glucose as a marker for glucose movements or by turbidimetry, which monitors glucose transport induced vesicle volume changes (Sen & Widdas, 1962; Carruthers & Melchior, 1983a). Initial rates determined by either turbidimetry or radiolabeled D-glucose are in excellent agreement. Indeed, the Michaelis constants for transfer obtained by kinetic analysis of initial rates of radiolabeled sugar flux or by integration of the time course of flux determined by turbidimetry are also in close agreement. The striking difference between methods lies in the magnitude of the cytochalasin B insensitive component of sugar flux. Figure 4 illustrates this point. Significant apparent leakage of labeled D-glucose occurs in the presence of cytochalasin B whereas turbidimetry indicates very low leakage of sugar from loaded vesicles. Similar results are obtained when $HgCl_2$ (0.75 mM) is used as an inhibitor of sugar flux. It is possible that the apparent leakage of radiolabeled D-glucose from LUVs results in part from nonspecific binding to vesicles. Direct evidence is not available, although protein-free egg lecithin LUVs display similar properties, having two phases of labeled D-glucose washout—rapid and slow. No such behavior is observed for D-glucose washout in egg lecithin LUVs when measured by turbidimetry (Carruthers & Melchior, 1983b).

D-Glucose penetrates or exits the reconstituted vesicles by two functional routes. The larger component of flux was inhibitable by cytochalasin B—a potent competitive inhibitor of sugar transport in both red cells and reconstituted systems formed by other procedures (see Jones & Nickson, 1981). The inhibition by cytochalasin B is saturable, with half-maximum inhibition produced by 0.5 μM inhibitor. This compares well with the apparent K_i for inhibition of hexose transfer in cells and reconstituted systems of approximately 0.2 μM (Jones & Nickson, 1981). The cytochalasin B sensitive component of glucose flux corresponds to the cytochalasin B sensitive, cytochalasin B binding protein mediated component of reconstituted sugar transport described here and in numerous earlier studies (Goldin & Rhoden, 1978; Kasahara & Hinkle, 1977; Wheeler & Hinkle, 1981). Over the range of unfractionated protein:lipid ratios used (1:6.6 to 1:66.6 by weight), hexose transfer activity was directly related to the protein:lipid ratio. With unfractionated membrane proteins, the specific transfer activity of the reconstituted system is 0.5 μmol·(mg of protein)$^{-1}$·min^{-1}. This compares with a native activity of 10 μmol·(mg of protein)$^{-1}$·min^{-1} (Jones & Nickson, 1981). With fractionated band 4.5 protein, hexose transfer activity was 5–8 μmol·mg^{-1}·min^{-1}, representing a 10–16-fold increase in the activity of the original membrane protein extract. This value for band 4.5 mediated transport in the reverse-phase evaporation system compares well with the values for band 4.5 mediated zero-trans and equilibrium-exchange uptake of glucose of 2 and 50 μmol·(mg of protein)$^{-1}$·min^{-1}, respectively, reported by Wheeler & Hinkle (1981).

We also observe significant transfer activity in vesicles containing band 3 proteins [0.43 μmol·(mg of protein)$^{-1}$·min^{-1}]. Kasahara & Hinkle (1977) had shown previously that band 3 proteins did not confer transport activity to synthetic membranes. We repeated the experiments using the above band 3 protein fraction, employing the reconstitution procedure of Kasahara & Hinkle (1977). Under these conditions we also observed no band 3 protein mediated hexose transfer activity. This seems not to arise from the sonication or freeze–thaw steps used by Kasahara and Hinkle, for inclusion of these steps prior to reconstitution by reverse-phase evaporation is without effect on the ability of the proteins to confer activity to the vesicles.

It is possible that the transfer activity supported by the band 3 protein fraction in the reverse-phase evaporation system results from contamination by band 4.5 proteins. Activity in the band 3 system is some 5–9% of activity supported by band 4.5. Moreover, what activity is present is inhibited by cytochalasin B—an inhibitor known to bind to band 4.5 protein (Baldwin et al., 1979). This would not, however, account for the inability of this protein fraction to support transport activity in membranes formed by the method of Kasahara and Hinkle—a method shown to support band 4.5 mediated transport.

These findings may have some bearing on the controversy surrounding the identities of the erythrocyte hexose transfer proteins (Jones & Nickson, 1981). Affinity labeling studies have shown that band 4.5 proteins bind the potent transport inhibitor cytochalasin B in a sugar-sensitive fashion (Baldwin et al., 1979) whereas band 3 proteins bind the potent inhibitor maltosyl isothiocyanate (MITC) in a maltose and cytochalasin B sensitive manner (Mullins & Langdon, 1980). The reconstitution studies of Kasahara & Hinkle (1977), however, identified only band 4.5 proteins as capable of confering transport activity (the physiological activity) to synthetic membranes. Our results confirm these earlier transport data but are also in agreement with the more recent studies of Shelton & Langdon (1983) showing that both protein fractions are capable of supporting hexose fluxes in synthetic membranes. Moreover, our data suggest that more than one reconstitution procedure may be necessary to determine the ability of a protein to express native function in synthetic membranes. A number of previous studies have been careful to point out that more than one protein may constitute the native transport system (Goldin & Rhoden, 1978; Jones & Nickson, 1978, 1981).

Registry No. Cytochalasin B, 14930-96-2; D-glucose, 50-99-7.

References

Baldwin, S. A., Baldwin, J. M., Gorga, F. R., & Lienhard, G. E. (1979) *Biochim. Biophys. Acta 552*, 183–188.

Baldwin, J. M., Gorga, J. C., & Lienhard, G. E. (1981) *J. Biol. Chem. 256*, 3685–3689.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965) *J. Mol. Biol. 13*, 238–245.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. 37*, 911–915.

Carruthers, A., & Melchior, D. L. (1983a) *Biochim. Biophys. Acta 728*, 254–266.

Carruthers, A., & Melchior, D. L. (1983b) *Biochemistry 22*, 5797–5807.

Düzgünes, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, S., James, T. L., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 732*, 289–299.

Garewal, H. S. (1973) *Anal. Biochem. 54*, 319–324.

Goldin, S. M., & Rhoden, V. (1978) *J. Biol. Chem. 253*, 2575–2583.

Hankin, B. C., Lieb, W. R., & Stein, W. D. (1972) *Biochim. Biophys. Acta 288*, 114–126.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 304-308.
Jones, M. N., & Nickson, J. K. (1978) *Biochim. Biophys. Acta 509*, 260-271.
Jones, M. N., & Nickson, J. K. (1981) *Biochim. Biophys. Acta 650*, 1-20.
Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 253*, 7384-7390.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Le Fevre, P. G. (1948) *J. Gen. Physiol. 31*, 405.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265-275.
Lukacovic, M. F., Feinstein, M. B., Sha'afi, R. I., & Perrie, S. (1981) *Biochemistry 20*, 3145-3151.
Maddy, A. H. (1982) *Red Cell Membranes. A Methodological Approach* (Ellory, J. C., & Young, J. D., Eds.) pp 44-46, Academic Press, New York.
Masaik, S. J., & Le Fevre, P. G. (1977) *Biochim. Biophys. Acta 465*, 371-377.
Mullins, R. E., & Langdon, R. G. (1980) *Biochemistry 19*, 1205-1212.
Sen, A. L., & Widdas, W. F. (1962) *J. Physiol. (London) 160*, 392-403.
Shelton, R. L., Jr., & Langdon, R. G. (1983) *Biochim. Biophys. Acta 733*, 25-33.
Szoka, F., Jr., & Papahadjopoulos, D. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 467-508.
Wheeler, T. J., & Hinkle, P. C. (1981) *J. Biol. Chem. 256*, 8902-8914.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324-332.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9170-9175.
Yu, J., Fischman, D. A., & Steck, T. L. (1973) *J. Supramol. Struct. 1*, 233-247.

Roles of Lipids and Proteins in the Ca^{2+}-PO_4-Induced Aggregation of Cytoskeleton-Free Erythrocyte Vesicle Membranes†

Kenneth S. Leonards‡ and Shinpei Ohki*

ABSTRACT: The roles of lipids and proteins in Ca^{2+}-PO_4-induced membrane aggregation were investigated. Cytoskeleton-free vesicles derived from intact human and rabbit erythrocytes ($H_E$ves and $R_E$ves, respectively) were employed as a model system. The $H_E$ves and $R_E$ves have a simplified membrane protein composition [band 3 proteins and glycoproteins PAS-1, -2, and -3 ($H_E$ves)] and normal lipid composition. Optimal experimental conditions for pH, [PO_4], and [$CaCl_2$] were determined for quantitatively examining the dynamics and extent of $H_E$ves and $R_E$ves aggregation, measured turbidimetrically. The aggregation process was found to be quite sensitive to small changes in pH and [PO_4] and much less sensitive to [$CaCl_2$]. The roles of membrane proteins in vesicle aggregation were examined by selectively modifying the proteins enzymatically. The roles of lipids were studied by using sonicated lipid vesicles [small unilamellar vesicles (SUVs)] made from Dodge ghost lipid extracts. Enzymatic treatment with trypsin, chymotrypsin, or Pronase had no effect on either the rates or the extent of vesicle aggregation (2-min incubation period). Neuraminidase treatment reduced both factors by approximately 20%. SUVs aggregated with Ca^{2+}-PO_4 in a way which depended on the PO_4/lipid ratio. Together the results suggest the following: (1) PO_4 is associated with the vesicle surface, involving the membrane lipids; (2) the vesicle + PO_4 incubation time component of the PO_4 effect is eliminated by enzymatically modifying the vesicle membrane proteins; (3) qualitative, rather than quantitative, properties of sialic acid containing molecules affect vesicle aggregation; and (4) with the exception of the incubation time effect, membrane proteins seem neither to promote nor to inhibit Ca^{2+}-PO_4-induced $H_E$ves or $R_E$ves aggregation.

The aggregation and fusion of membranes are two of the most basic processes in biology, being fundamentally involved in such phenomena as exocytosis, plasmogamy, and karyogamy. Of particular interest are the possible roles of membrane lipids and proteins in these processes. Recently, researchers have turned to the use of erythrocytes and/or erythrocyte ghosts as a model system for examining membrane aggregation and fusion. Although erythrocytes do not normally fuse in nature, their relative simplicity, ready availability, and well-characterized chemical composition strongly recommend their use. As a result, it has been demonstrated that various chemical agents, such as oleoylglycerol (Quirk et al., 1978; Blow et al., 1979), lysolecithin (Lucy, 1976), calcium-phosphate (Zakai et al., 1976, 1977; Majumdar & Baker, 1980), poly(ethylene glycol) (Ahkong et al., 1975; Knutton, 1979a,b), and inactivated Sendi virus (Peretz et al., 1974; Volsky & Loyter, 1978; Knutton, 1979a,b; Lalazar & Loyter, 1979; Sekiguchi et al., 1981), will induce the aggregation and fusion of erythrocyte membranes.

These studies, however, still suffer from a number of problems in data interpretation, due mainly to the still large heterogeneity in their membrane composition, especially proteins, and the difficulty in distinguishing between phenomena involving cytoskeleton–membrane interactions and those involving the lipids and/or proteins of the membrane proper. To help alleviate these problems, we have developed a simple method for obtaining large ($\simeq$0.5-1.0 μm), cytosk-

† From the Department of Biophysical Sciences, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214.
Received June 14, 1983; revised manuscript received January 11, 1984. This work was supported by a grant from the National Institutes of Health (GM24840).
‡ Present address: Department of Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22908.

0006-2960/84/0423-2718$01.50/0