Stein, W. D. (1967) *The Movement of Molecules Across Cell Membranes*, Academic Press, New York.
Taverna, R. D., & Langdon, R. G. (1973a) *Biochim. Biophys. Acta 298*, 412.
Taverna, R. D., & Langdon, R. G. (1973b) *Biochim. Biophys. Acta 323*, 207.
Taverna, R. D., Langdon, R. G. (1973c) *Biochem. Biophys. Res. Commun. 54*, 593.
Tokes, Z. A., & Chambers, S. M. (1975) *Biochim. Biophys. Acta 389*, 325.
Trosper, T., & Levy, D. (1977) *J. Biol. Chem. 252*, 181.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Wilson, D. B. (1978) *Annu. Rev. Biochem. 47*, 933.
Wolfrom, M. L., & Miller, J. B. (1956) *Anal. Chem. 28*, 1037.
Wolfrom, M. L., & Thompson, A. (1962) *Methods Carbohydr. Chem. 1*, 334.
Zala, C. A., & Kahlenberg, A. (1976) *Biochem. Biophys. Res. Commun. 72*, 866.
Zoccoli, M. A., Baldwin, S. A., & Lienhard, G. E. (1978) *J. Biol. Chem. 253*, 6923.

Maltosyl Isothiocyanate: An Affinity Label for the Glucose Transporter of the Human Erythrocyte Membrane. 2. Identification of the Transporter†

Richard E. Mullins‡ and Robert G. Langdon*

ABSTRACT: Maltosyl isothiocyanate (MITC), a potent irreversible inhibitor of glucose transport in human erythrocytes [Mullins, R. E., & Langdon, R. G. (1980) *Biochemistry* (preceding paper in this issue)], has been found to react almost exclusively with band 3 of the human erythrocyte membrane. The incorporation of [^{14}C]MITC into band 3 was found to be antagonized by transportable sugars or competitive inhibitors of transport. On the basis of [^{14}C]MITC incorporation into band 3 and MITC inhibition of transport, it is estimated that there are 3×10^5 glucose transporters present in the erythrocyte membrane. It was found that [^{14}C]MITC-labeled band 3 could be converted into ^{14}C-labeled band 4.5 during the Triton X-100 extraction procedure described by Kasahara & Hinkle [Kasahara, M., & Hinkel, P. C. (1977) *J. Biol. Chem. 252*, 7384]. On the basis of the evidence presented here and in the preceding paper, it is suggested that in the native erythrocyte membrane a component of band 3 is the glucose transport protein and that during purification with nonionic detergents the transport protein may be enzymatically degraded with some retention of activity.

During recent years there have been several efforts to identify the glucose transporter in the human erythrocyte membrane. There have been two general approaches to this question of carrier identity. One approach has been to isolate and purify different membrane protein components and incorporate them into synthetic membranes in an attempt to reconstitute D-glucose-specific translocation. Another approach has been to devise an affinity label for the glucose transport protein and to label the carrier while in its native state in the membrane. Investigators using these techniques have been led to different conclusions on the question of carrier identity.

Taverna & Langdon (1973b) reported that the glucose transporter was probably a component of band 3 [nomenclature of Fairbanks et al. (1971)] based on their data using D-glucosyl isothiocyanate as an affinity label. Kasahara & Hinkle (1977) have reported that band 4.5, a 55 000 M_r protein component of the membrane, is the apparent carrier from their reconstitution experiments. Jones & Nickson (1978), on the other hand, using reconstitution into planar black lipid membranes, have reported that band 3 is the most likely candidate to be the glucose carrier. Other investigators have presented evidence supporting either band 3 (Trosper & Levy, 1977; Phutrakul & Jones, 1979) or band 4.5 (Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978; Batt et al., 1976; Abbot & Schachter, 1976; Zoccoli et al., 1978) as the carrier. In this paper we present evidence that may reconcile the observations of all investigators.

In the preceding paper (Mullins & Langdon, 1980) a kinetic analysis of maltosyl isothiocyanate (MITC)[1] inhibition of glucose transport in human erythrocytes was reported. MITC fulfilled the kinetic criteria for an affinity label of the glucose transport system proposed by Groman et al. (1977).

In this communication we show that [^{14}C]MITC combines covalently in a stoichiometric reaction with a component of band 3, and this combination is specifically antagonized by transported sugars or competitive inhibitors of transport; this fulfills the final requirement of an affinity label. This label can therefore be used to identify the glucose carrier.

We propose on the basis of evidence presented here and in the preceding paper that band 3 in the native erythrocyte membrane contains the glucose transport protein and that during the nonionic detergent extractions used by some investigators to purify the putative membrane transporter a 55 000-dalton fragment which migrates with band 4.5 is generated from band 3 by the action of neutral proteases associated with erythrocyte membranes. This band 4.5

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 20, 1979.* This work was supported by Research Grant GM-19319 from the National Institutes of Health.

‡ Material for this publication is from a dissertation submitted by R.E.M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, University of Virginia.

[1] Abbreviations used: MITC, maltosyl isothiocyanate; PBS, phosphate-buffered saline; glycine–PBS, 100 mM glycine dissolved in PBS, pH 8; GOC ghosts, glucose oxidase-catalase containing ghosts; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; FDNB, fluorodinitrobenzene; D-GITC, glucosyl isothiocyanate; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/80/0419-1205$01.00/0

fragment of band 3 apparently partially retains the transport function and is easily reincorporated into synthetic membranes, thus leading to its tentative identification as the glucose transport protein by several investigators. This view has also been expressed recently by Phutrakul & Jones (1979).

Experimental Procedures

Materials

[^{14}C]Maltose (360 μCi/μmol) was purchased from ICN. Maltose monohydrate, grade II, α-chymotrypsin, Type II, Coomassie Brilliant Blue, Triton X-100, Triton X-114, sodium dodecyl sulfate ($NaDodSO_4$), and Trizma base were purchased from Sigma Chemical Co. Acrylamide and bis(acrylamide) were purchased from Aldrich Chemical Co. *N,N,N′,N′*-Tetraethylenediamine was supplied by Eastman. Sil G precoated silica gel plates were purchased from Brinkmann Instrument Inc. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was purchased from Polyscience. 2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) were purchased from Amersham/Searle. Filmware scintillation counting bags were purchased from Nalge Co. Bio Beads SM-2 were supplied by Bio-Rad Laboratories.

Methods

$NaDodSO_4$ was recrystallized from 95% ethanol. Acrylamide and *N,N′*-methylenebis(acrylamide) were recrystallized from acetone. Maltose monohydrate was purified via the octaacetate as previously described (Mullins & Langdon, 1980). To form [^{14}C]MITC, we diluted [^{14}C]maltose with unlabeled pure maltose to give a final specific activity of 1–4 μCi/μmol and synthesized [^{14}C]MITC as previously reported (Mullins & Langdon, 1980).

Fresh human blood was collected by venipuncture; NaEDTA, 1 mg/mL of blood, was used as an anticoagulant. The cells were collected by centrifugation and they were washed free of serum and buffy coat by repeated centrifugation at 3500*g* for 10 min in 150 mM NaCl and 5 mM sodium phosphate, pH 8 (PBS). Great care was taken to obtain an erythrocyte preparation free from leukocytes, which are known to possess large amounts of proteases.

Zero trans glucose uptake was measured as described by Mullins & Langdon (1980).

Preparation of [^{14}C]MITC-Labeled Erythrocyte Membranes. To a 50% suspension of erythrocytes in either PBS or PBS plus protecting agent at 37 °C, sufficient 45 mM [^{14}C]MITC was added to give the desired concentration. The reaction mixture was mixed throughly and maintained at 37 °C with occasional gentle agitation. Reaction was terminated by washing the cells twice with 50 volumes of 100 mM glycine, 150 mM NaCl, and 5 mM sodium phosphate, pH 8 (glycine–PBS) and 3 times with 50 volumes of PBS at 25 °C.

Membranes of the labeled cells were isolated by the method of Fairbanks et al. (1971). Packed erythrocytes were lysed by the addition of 100 volumes of 5 mM sodium phosphate, pH 8 (5P8), at 4 °C. This was centrifuged at 15000*g* for 20 min. The membrane pellet was washed with 5P8 until no hemoglobin was observed in the supernatant. The button of unlysed cells and debris was aspirated after each wash and discarded.

Triton X-100 extractions were performed as described by Kasahara & Hinkle (1977). External α-chymotrypsin digestion of erythrocytes was performed as described by Steck et al. (1978). Protein concentrations were determined by the method of Lowry et al. (1951).

Membrane suspensions were prepared for electrophoresis by making them 2% in $NaDodSO_4$ and 1.0% in dithiothreitol and heating at 100 °C for 1 min. They were then electrophoresed on 7.5% polyacrylamide disc gels as described by Laemmli (1970). Radioactivity in the various protein species was determined by sectioning the gels into disks 2 mm thick and placing each disk into a Filmware bag. The disks were crushed manually and 0.3 mL of 6 N HCl was added to each bag. The samples were then heated to 100 °C for 1 h and allowed to cool. The HCl was removed under reduced pressure in a vacuum desiccator containing NaOH pellets. After the samples were dried, 0.3 mL of water was added to each bag, followed by 3.0 mL of a scintillation cocktail composed of 75% toluene, 25% Triton X-114, 3 g/L PPO, and 0.2 g/L POPOP. The bags were then sealed and counted in a Beckman LS-233 liquid scintillation counter. Gels were stained as described by Fairbanks et al. (1971). Densitometric scans were made by using an EC 910 transmission densitometer with a second-order interference filter.

Results

Previous kinetic experiments (Mullins & Langdon, 1980) demonstrated that MITC was a covalent inhibitor of glucose transport which had properties expected of an affinity label. It was therefore of interest to determine with which membrane proteins it reacted and whether its covalent attachment could be specifically prevented by those agents which protected against transport inhibition.

To demonstrate incorporation of [^{14}C]MITC into the proteins of the erythrocyte membrane, we incubated [^{14}C]MITC with intact erythrocytes or with pink ghosts under conditions (9 mM MITC; 10 min; 37 °C) which had been found in kinetic experiments to inhibit transport ~50%. Well-washed membranes were prepared and duplicate aliquots were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. One gel from each set was fixed and stained with Coomassie Brilliant Blue, while the others were sliced into 2-mm segments and their ^{14}C content was measured as described under Methods. There was no alteration in the electrophoretic pattern of stainable proteins from erythrocyte membranes which had been labeled with MITC under the conditions used in the subsequent experiments. In Figure 1 is shown the distribution of Coomassie Brilliant Blue stained protein and radioactivity observed in a typical experiment when erythrocytes were treated with [^{14}C]MITC. In this experiment over 95% of the radioactivity applied to the gel was recovered in the gel slices. It is strikingly evident that of the 530 dpm present in the gel, 486 dpm was recovered with a protein component of band 3 which appeared to have M_r of about 100000 while less than 10% of the radioactivity was present in band 4.5. These values correspond to the presence of 2.2×10^5 maltosyl residues per cell in band 3 while 2.3×10^4 residues per cell were present in band 4.5. In a series of experiments, recovery of radioactivity in band 3 ranged from 79 to 95% of the total, and the average was 88%. No radioactivity was found in the soluble proteins released by lysis of the erythrocytes. The kinetic experiments had demonstrated that maltose, mannose, and cytochalasin B each protected against transport inhibition by MITC, and it seemed imperative to demonstrate that incorporation of [^{14}C]maltosyl units into the transporter could also be inhibited by transported sugars and by specific inhibitors of monosaccharide transport. When D-glucose, maltose, or cytochalasin B were present during incubation of erythrocytes with [^{14}C]MITC, the results shown in Table I were obtained. It is quite evident that both sugars and cytochalasin B substantially reduced incorporation of label into band 3, although protection was far from complete. Sucrose and L-glucose were entirely without effect. It

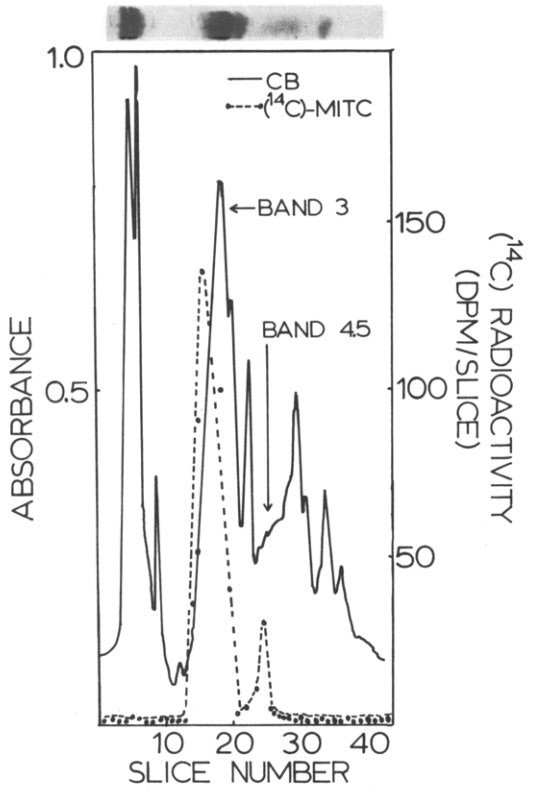

FIGURE 1: Distribution of proteins and radioactivity in membranes from erythrocytes labeled with [^{14}C]MITC. A 50% suspension of washed erythrocytes in PBS was made 9 mM in [^{14}C]MITC and incubated at 37 °C for 10 min, conditions which lead to 45–50% inhibition of glucose transport. The reaction was terminated by two washes with 50 volumes of glycine–PBS, followed by three washes with PBS as described under Methods. White ghosts were prepared and solubilized in 2% $NaDodSO_4$ and 1% dithiothreitol and heated for 1 min at 100 °C before electrophoresis on 7.5% polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue (Fairbanks et al., 1971) and scanned in a densitometer. A duplicate gel cylinder was sliced into disks 2 mm thick, and the radioactivity in each disk was determined as described under Methods. (—) Coomassie Brilliant Blue staining intensity; (---) ^{14}C dpm/slice.

should be particularly noted that the very small amount of label incorporated into band 4.5 was, within experimental error, unchanged by the presence of glucose or maltose. In a series of four identical experiments, entirely similar results were obtained.

It was evident that the extent of protection by maltose against incorporation of [^{14}C]MITC into band 3 was less than that afforded against inhibition of transport (Mullins & Langdon, 1980). Therefore, a series of experiments were carried out in which both inhibition of transport by MITC and incorporation of [^{14}C]MITC into band 3 were measured under identical conditions. The results of five separate experiments are presented in Figure 2. It is evident that the extent of incorporation of [^{14}C]MITC into band 3 was proportional to the degree of inhibition of transport when MITC was reacted with cells for different times (×) without including a protecting agent. More important, however, is that when either maltose (○) or cytochalasin B (△) was included in the incubation, the degree of inhibition and [^{14}C]MITC incorporation fall on the same line as treatment with MITC alone even though transport inhibition ranged from 10 to 80% of control values. This strongly suggests that the site being labeled by MITC was indeed a site occupied by maltose and made inaccessible by cytochalasin B, which is presumed to be a glucose binding site. By extrapolation of the line to the ordinate intercept, it appears that approximately 8.8×10^4 maltosyl residues could be incorporated into band 3 per cell without affecting transport. Extrapolation to 100% inhibition reveals that transport was completely inhibited when 3.9×10^5 maltosyl residues per cell had been incorporated into band 3. Subtraction of 8.8×10^4 from this number suggests that there are approximately 3.0×10^5 band 3 sites per cell which react with MITC and which are related to glucose transport. This is in very good agreement with the number of glucose transport sites reported by other investigators (Taverna & Langdon, 1973a,b; Lienhard et al., 1977). The fact that MITC was incorporated into transport

Table I: Incorporation of [^{14}C]MITC into Proteins of Erythrocyte Membranes in the Presence or Absence of Protecting Agents[a]

labeling conditions	incorpn of [^{14}C]MITC into protein (dpm/μg of protein) band 3	band 4.5	maltosyl residues incorpd per cell ($\times 10^{-5}$)[b] band 3	band 4.5
[^{14}C]MITC	1.82	0.25	1.97	0.26
[^{14}C]MITC plus 150 mM maltose	1.32	0.14	1.43	0.15
[^{14}C]MITC plus 150 mM D-glucose	1.07	0.21	1.16	0.22
[^{14}C]MITC plus 10 μM cytochalasin B	1.23	0.22	1.30	0.23
[^{14}C]MITC plus 150 mM L-glucose	1.91	0.23	2.07	0.24
[^{14}C]MITC plus 150 mM sucrose	1.80	0.24	1.95	0.26

[a] A 50% suspension of washed erythrocytes in PBS or PBS plus protecting agent was incubated at 37 °C for 5 min. The suspension was made 9 mM in [^{14}C]MITC (1.80 μCi/μmol) by the addition of a freshly prepared stock solution of [^{14}C]MITC. The reaction was terminated after 10 min, the cells were washed, their membranes were isolated and electrophoresed, and the distribution of radioactivity was determined as in Figure 1. [b] The number of maltosyl residues per cell was determined by using 1.39×10^6 cells/μg of membrane protein as determined by Dodge et al. (1963).

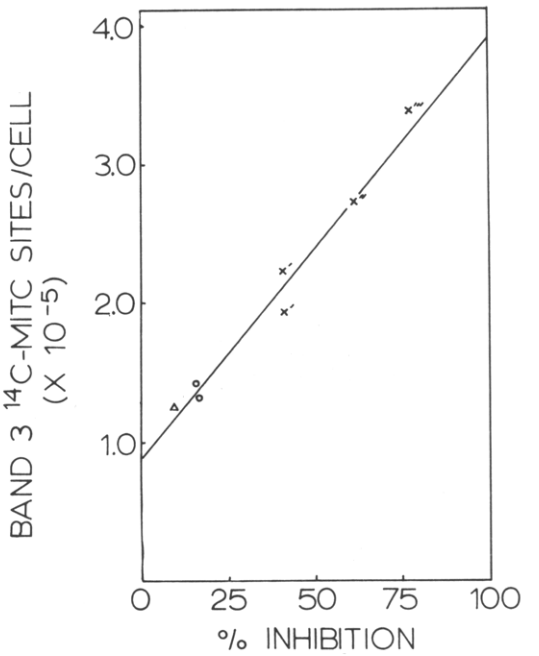

FIGURE 2: Relationship between [^{14}C]MITC incorporation into band 3 and inhibition of glucose transport. Erythrocytes were treated at 37 °C with 9 mM MITC alone for 10 (×′), 20 (×″), and 30 min (×‴) or in the presence of 150 mM maltose for 10 min (○) or in the presence of 10 μM cytochalasin B for 10 min (△). The number of [^{14}C]maltosyl residues incorporated into band 3 per cell was determined as described in Table I. Glucose transport rate was measured as described under Methods and compared with the rate obtained with an equal number of untreated cells.

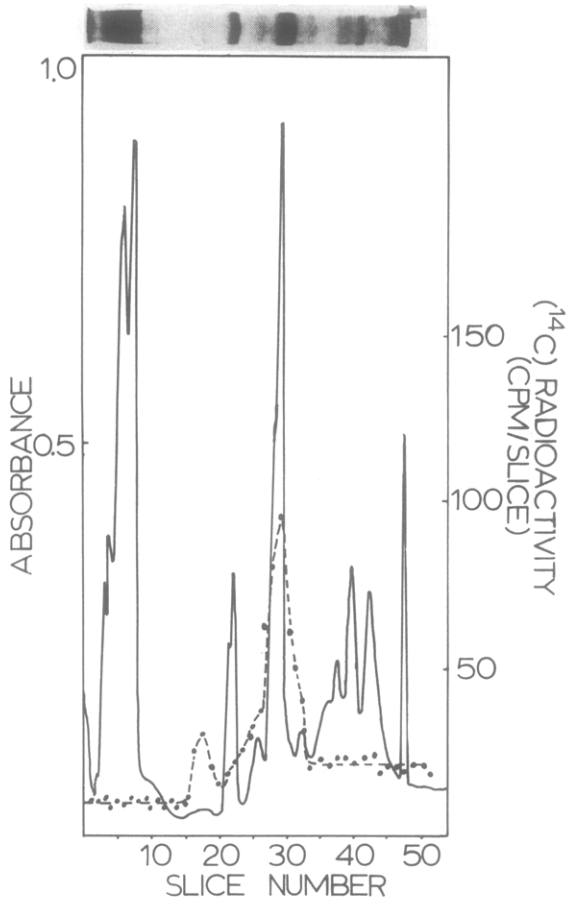

FIGURE 3: Effect of external chymotryptic digestion on distribution of radioactivity in membrane proteins of erythrocytes which had been labeled with [^{14}C]MITC. Erythrocytes were labeled with [^{14}C]MITC as described in Figure 1. The cells were digested overnight at room temperature with α-chymotrypsin, 100 μg/mL (Steck et al., 1978). The digested cells were washed extensively, converted to white ghosts, and prepared for electrophoresis. Solubilized membranes were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis and were analyzed for protein and ^{14}C as described in Figure 1. (—) Coomassie Brilliant Blue staining intensity; (●--●) ^{14}C cpm/slice.

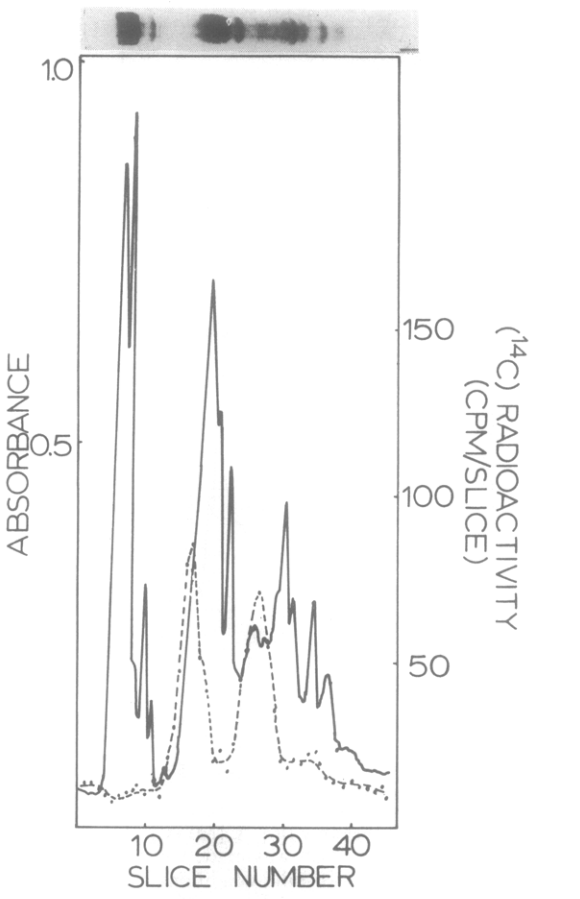

FIGURE 4: Autodigestion of erythrocytes which had been labeled with [^{14}C]MITC. Erythrocytes were treated with [^{14}C]MITC as described in Figure 1. The labeled cells were incubated for 36 h at 25 °C with occasional mixing before their membranes were isolated and prepared for electrophoresis. Membranes were solubilized, electrophoresed, and analyzed for protein and radioactivity as described in Figure 1. (—) Coomassie Brilliant Blue staining intensity; (●--●) radioactivity, cpm/slice.

unrelated sites accounts for the incomplete protection observed in Table I.

To reconcile these results which point to band 3 as the transport protein with a mass of evidence which suggests that band 4.5 acts as the transporter, we investigated the possible conversion of band 3 to band 4.5 under various conditions. It seemed possible that band 4.5 might arise from band 3 as a result of enzymatic degradation. Steck et al. (1978) have shown that external proteolysis of erythrocytes by α-chymotrypsin splits band 3 into a 55 000-dalton integral, transmembrane component which migrates in NaDodSO$_4$–polyacrylamide gel electrophoresis in the band 4.5 region as well as a 38 000-dalton fragment which is also integral.

Results similar to those of Steck et al. (1978) are illustrated in Figure 3 where NaDodSO$_4$–polyacrylamide gel electrophoresis gels of membranes from [^{14}C]MITC-labeled erythrocytes which have undergone external chymotryptic digestion are shown. It is apparent that the only changes demonstrable by Coomassie Brilliant Blue staining of the proteins of membranes from these cells are the disappearance of band 3 and the appearance of a 55 000 M_r fragment migrating in the band 4.5 region and a 38 000 M_r fragment, which is not always easily identified.

This figure also shows the distribution of radioactivity in the membrane proteins of these cells. It would be expected that if the protein originally labeled were band 3, the ^{14}C-labeled chymotryptic fragment would migrate either in the band 4.5 region or in the region corresponding to a M_r of 38 000 (above band 6). This was indeed the case; approximately 75% of the label in the membranes from α-chymotrypsin-digested cells migrated in the band 4.5 region, while only 25% of the label was retained in the band 3 region. No radioactivity was found in the 38 000 M_r region.

In a similar experiment, designed to examine the endogenous conversion of labeled band 3 to smaller labeled products, cells were labeled with [^{14}C]MITC. Membranes prepared from one aliquot of cells were solubilized immediately by addition of NaDodSO$_4$ to 2% and heating to 100 °C for 1 min; they were then stored at −20 °C until used as an electrophoretic standard. Another portion of the labeled cells was incubated at 25 °C for 36 h in PBS before preparing membranes and solubilizing by the usual procedure. A third aliquot was incubated at 25 °C for 36 h in the presence of 100 μM phenylmethanesulfonyl fluoride. Figure 4 shows the stained polyacrylamide gel pattern and the distribution of radioactivity among the membrane proteins from cells which had been incubated at 25 °C for 36 h. A substantial increase in label was found in the band 4.5 region compared to control ghosts. The amount of label in this region increased from less than 10% in the control to almost 40% in the membranes from self-digested cells. This was accompanied by a corresponding decrease of radioactivity found in band 3. The distribution

of radioactivity in the membranes from cells incubated in the presence of phenylmethanesulfonyl fluoride was identical with that of the unincubated control. A slight increase in Coomassie Brilliant Blue staining intensity was noted in the band 4.5 region of incubated cells. Quantitative comparisons were made from densitometric scans of Coomassie Brilliant Blue stained gels using a compensating polar planimeter to measure total areas and the areas under each peak. These showed that the area under band 4.5 increased from 4% of the total area in control membranes to 11% of the total area in membranes from cells incubated in PBS for 36 h. The membranes from cells incubated with PMSF addition were identical with those of the unincubated control. These experiments established that erythrocytes, without added proteases, have the ability under certain conditions to convert a portion of band 3 into a band 4.5 component. Since this conversion was inhibited by PMSF, it was probably proteolytic.

It has been reported that neutral proteases are associated with erythrocyte membranes (Bernacki & Bosmann, 1972; Tokes & Chambers, 1975) and that proteolytic activity is extracted and activated with nonionic, nondenaturing detergents (Tokes & Chambers, 1975). We therefore thought it desirable to determine if this activity was capable of converting [^{14}C]MITC-labeled band 3 into labeled band 4.5 under conditions used by several investigators to isolate the band 4.5 peptide used in reconstituting D-glucose-specific transport activity. To examine this possibility, we converted erythrocytes labeled with [^{14}C]MITC to white ghosts and prepared a control aliquot for electrophoresis by solubilizing it in NaDodSO$_4$–dithiothreitol and freezing it until it was used as an electrophoretic standard. The remainder of the [^{14}C]MITC-labeled membranes were treated according to the procedure of Kasahara & Hinkle (1977), which uses a low ionic strength wash of the membranes, followed by a high ionic strength wash, and finally an extraction of the membrane residue with Triton X-100, a nondenaturing, nonionic detergent, to yield a soluble fraction plus an insoluble residue. As shown in Figure 5 the soluble portion contains bands 3 and 4.5. Illustrated is the distribution of ^{14}C in the proteins of the Triton X-100 soluble fraction, which contained 89% of the total radioactivity in the ghosts. The Triton X-100 residue derived from [^{14}C]MITC-labeled cells contained the remainder of the radioactivity. It is evident that much more radioactivity migrated in the band 4.5 region of the Triton X-100 soluble fraction, compared to the electrophoretic pattern of the unextracted white ghosts prepared from [^{14}C]MITC-labeled erythrocytes (Figure 1). From four experiments, the average distribution of total ^{14}C label between bands 3 and 4.5 in white ghosts prior to the Kasahara and Hinkle procedure was 88% in band 3 and 6% in band 4.5; the range was 79–95% for band 3 and 4–9% for band 4.5. In the soluble Triton extract, the average distribution of radioactivity in three experiments was 49% (range 45–55%) in band 3 and 44% (range 39–51%) in band 4.5.

A summary of the data from a typical experiment is found in Table II. It can be seen by comparing the distribution of radioactivity in control membranes and membranes extracted with Triton X-100 that all of the radioactivity initially present in the membranes prepared from ^{14}C-labeled erythrocytes was recovered in the Triton X-100 extract plus pellet. Since the radioactivity applied to each gel was recovered almost quantitatively in bands 3 and 4.5, the increased ratio of radioactivity in band 4.5/band 3 cannot have resulted from selective extraction of band 4.5 but must have resulted from an absolute increase in this component at the expense of band 3. The same argument excludes the possibility that radioactivity was lost

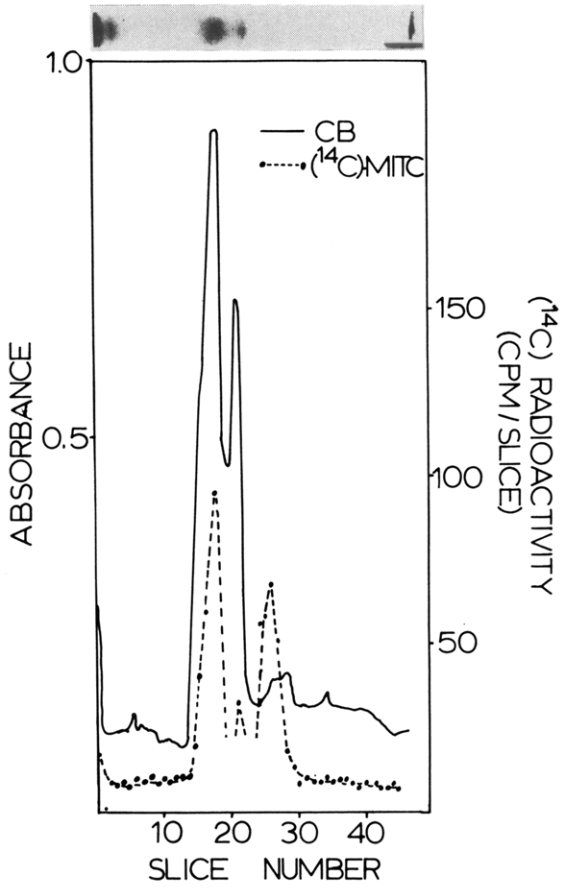

FIGURE 5: Distribution of membrane proteins and radioactivity in [^{14}C]MITC-labeled erythrocyte membranes after Triton X-100 extraction. Erythrocytes were treated with [^{14}C]MITC as described in Figure 1; their membranes were isolated and treated to produce a soluble preparation enriched in bands 3 and 4.5 (Kasahara & Hinkle, 1977). The membranes were incubated for 20 min at 37 °C in 10 volumes of 0.1 M EDTA and 0.1 M Tris-HCl, pH 8.0. After sedimentation, the pellet was washed once in the same solution and incubated with 3 volumes of 0.5 M NaCl and 5 mM Tris-H_2SO_4, pH 7.4, for 20 min at 4 °C. The membranes were sedimented and washed once with the high ionic strength solution and once with 15 volumes of 10 mM Tris-H_2SO_4, pH 7.4. The pellet was mixed with an equal volume of 0.5% Triton X-100 in 10 mM Tris-H_2SO_4, pH 7.5, and allowed to stand 20 min at 4 °C. After centrifugation at 100000g for 60 min at 4 °C, the supernatant fraction was treated with Bio Beads SM-2 (Holloway, 1973) for 24 h at 4 °C. The sample was then prepared for electrophoresis as usual; the Triton X-100 soluble fraction was analyzed for distribution of protein and of radioactivity as described in Figure 1.

selectively from band 3 into some component other than band 4.5. In the membranes from cells digested with α-chymotrypsin and cells incubated at 25 °C for 36 h, some radioactivity was lost as shown in Table II. Radioactivity was found in the supernatant of these cell suspensions, but no attempt was made to ascertain if it remained attached to a peptide fragment. Under both of these conditions, however, both the total radioactivity and the specific radioactivity of the protein in the band 4.5 region increased, indicating a net increase of band 4.5 radioactivity at the expense of radioactivity that originally migrated as band 3.

There is very extensive evidence which indicates that the erythrocyte membrane anion transporter is a component of band 3 (Rothstein et al., 1976; Cabantchik & Rothstein, 1974a; Ho & Guidotti, 1975; Ross & McConnell, 1977). It therefore became of interest to determine whether covalent affinity labels for the anion transporter would inhibit the incorporation of [^{14}C]MITC into band 3. Therefore, eryth-

Table II: Conversion of [^{14}C]MITC-Labeled Band 3 to [^{14}C]MITC-Labeled Band 4.5[a]

source of membranes	[^{14}C]MITC incorpn (dpm/μg of protein) band 3	band 4.5	maltosyl residues incorpd per ghost ($\times 10^{-5}$)[b] band 3	band 4.5
(A) [^{14}C]MITC-labeled cells	2.49	0.29	1.90	0.22
(B) [^{14}C]MITC-labeled cells incubated 36 h at 25 °C	1.36	0.94	1.03	0.72
(C) [^{14}C]MITC-labeled cells incubated 24 h at 25 °C with α-chymotrypsin	0.58	1.58	0.44	1.20
(D) [^{14}C]MITC-labeled cells whose ghosts were extracted with Triton X-100[c]				
supernatant	1.31	1.32	0.99	1.00
residue	0.30	0	0.23	0

[a] A 50% suspension of washed erythrocytes in PBS was labeled with [^{14}C]MITC (2.56 μCi/μmol), and the reaction was quenched as described in Figure 1. One aliquot of cells (A) was converted to white ghosts, prepared for electrophoresis, and stored at −20 °C until it was used as an electrophoretic standard. Aliquots B and C were incubated as 50% suspensions in PBS in the absence or presence of α-chymotrypsin (100 μg/mL) for 36–24 h, respectively, at 25 °C as described by Steck et al. (1978). The cells were then washed, and the isolated membranes were subjected to electrophoresis. Aliquot D was converted into white ghosts and extracted with EDTA, NaCl, and Triton X-100 as described by Kasahara & Hinkle (1977). The resulting supernatant and pellet were subjected to electrophoresis. Another aliquot of cells was converted to white ghosts and electrophoresed, and the gel was stained with Coomassie Brilliant Blue as a standard. Gels were analyzed for radioactivity as described in Figure 1. [b] The number of maltosyl residues per cell was determined by using 1.39×10^6 cells/μg of membrane protein as determined by Dodge et al. (1963). [c] As described by Kasahara & Hinkle (1977).

Table III: Incorporation of [^{14}C]MITC into Band 3 of Erythrocytes Pretreated with DIDS[a]

expt	[^{14}C]MITC plus cells (dpm/μg of protein)	[^{14}C]MITC plus cells pretreated with DIDS (dpm/μg of protein)
1	2.96	2.32
2	1.77	1.62
3	2.51	2.11

[a] A 10% suspension of washed erythrocytes in PBS (10 mL) was made 20 μM in DIDS by the addition of 10 μL of a stock solution of DIDS in 50% ethanol. The cells were incubated with occasional gentle agitation for 30 min at 37 °C. They were then washed several times in PBS before being treated with [^{14}C]MITC in the usual manner. The radioactivity associated with band 3 was determined as described in Figure 1. Control gels were run of membranes from cells treated with [^{14}C]MITC but not pretreated with DIDS.

rocytes were preincubated with diisothiocyanostilbenedisulfonate (DIDS), an extremely powerful covalent affinity label for the anion transporter, under conditions which completely inhibit anion translocation and which result in the incorporation of approximately 10^6 molecules of labeled DIDS into the erythrocyte membrane (Lepke et al., 1976; Ship et al., 1977). Following this pretreatment, the cells were washed and incubated with [^{14}C]MITC. The results, presented in Table III, show that DIDS pretreatment decreased [^{14}C]MITC incorporation into band 3 to a relatively minor extent.

Discussion

Several major approaches to identifying the erythrocyte membrane glucose transporter have been employed. Broadly, these are (1) the use of affinity labels, (2) the use of selective extraction techniques, and (3) extraction and purification of membrane proteins, followed by attempts to reconstitute glucose transport by incorporation of these proteins into lipid bilayers. The results of these different approaches have often led to different conclusions.

Taverna & Langdon (1973b) synthesized D-glucosyl isothiocyanate (GITC) as an affinity label for the glucose transporter; they concluded from their data that band 3 might contain the glucose transporter and that band 4 might also be involved. It was found that there were about 300 000 GITC-reactive sites per cell in band 3 which were protected by glucose; this number was approximately the same as the number of high-affinity cytochalasin B binding sites in erythrocyte membranes (Taverna & Langdon, 1973a; Lin & Spudich, 1974). Similar results were obtained by Trosper & Levy (1977) with rat adipocytes; they found that the photolabile compound *N*-(4-azido-2-nitrophenyl)-2-aminodeoxy-D-glucose labeled two membrane proteins having molecular weights of approximately 100 000 and 81 000. On the other hand, Jung & Carlson (1975) reported that glucose-enhanced incorporation of fluorodinitrobenzene led to labeling of a 180 000-dalton erythrocyte membrane protein, while Batt et al. (1976) reported results obtained with impermeant maleimides which strongly suggested that an erythrocyte membrane component of ~65 000 M_r migrating in the band 4.5 region was implicated in glucose transport. Zoccoli & Lienhard (1977) concluded on the basis of Cu^{2+}/orthophenanthroline oxidation of band 3 that this component was not involved in glucose transport.

Cytochalasin B is a tightly bound reversible inhibitor of glucose translocation (Taverna & Langdon, 1973a; Lin & Spudich, 1974; Lin & Snyder, 1977; Lin & Lin, 1978; Jung & Rampal, 1977; Pinkofsky et al., 1978). This property has been utilized by Lienhard and co-workers (Lienhard et al., 1977; Zoccoli et al., 1978) in combination with differential extraction and FDNB labeling to attempt to identify the glucose transporter. They have also concluded that this is probably present in band 4.5.

Attempts to identify the glucose transporter by the techniques of differential extraction of membranes or by extraction and purification of erythrocyte membrane proteins followed by reconstitution of glucose transport by incorporation of these proteins into lipid bilayer systems have also led to conflicting results. Kasahara & Hinkle (1976) as well as Zala & Kahlenberg (1976) and Kahlenberg (1976) initially reported that partial purification of Triton X-100 extracts of membranes or DMMA-extracted membranes yielded band 3 preparations which were active in reconstituting glucose transport in lipid vesicles and in glucose binding (Kahlenberg et al., 1971), while Zoccoli & Lienhard (1977) concluded from differential extraction studies that bands 1, 2, 5, and 6 could be excluded as containing the transporter. Jones & Nickson (1978) have also reported that band 3 preparations successfully reconstitute glucose transport in planar bilayer systems. However, Kasahara & Hinkle (1977) as well as Kahlenberg & Zala (1977) have retracted their initial claims that band 3 mediates glucose transport and have instead concluded that band 4.5 contains the transporter. A similar conclusion has been reached by Goldin & Rhoden (1978). It is thus evident that there has been considerable uncertainty concerning the identity of the transporter based upon reconstitution studies.

The data presented here and in the preceding paper (Mullins & Langdon, 1980) clearly show that maltosyl isothiocyanate satisfies the criteria (Groman et al., 1977) for an affinity label of the glucose transporter, and they strongly suggest that this reagent combines covalently with the transporter at a sugar binding site. Maltose itself is a substrate analogue with a K_I of approximetly 14 mM; this is very close to the K_T for zero trans influx of glucose, which is approximately 10 mM (Taverna & Langdon, 1973a). Furthermore MITC, prior to covalent attachment, associates reversibly with the transporter with a half-saturation constant of 9–16 mM (Mullins & Langdon, 1980). Inclusion of transport substrates or inhibitors of transport protected against transport inactivation by MITC, while substances such as L-glucose and sucrose did not. When [^{14}C]MITC was reacted with erythrocytes or sealed ghosts, ~90% of the label incorporated into membrane proteins was localized in band 3, and the extent of incorporation of [^{14}C]MITC into band 3 was strictly proportional to the extent of transport inhibition (Figure 2). Furthermore, transport substrates such as D-mannose and D-glucose as well as reversible inhibitors of monosaccharide transport such as maltose and cytochalasin B protected against both transport inhibition and [^{14}C]MITC incorporation into band 3 to the same extent. The data in Figure 2 reveal that there was a small amount (~90 000 residues per cell) of incorporation of [^{14}C]MITC into band 3 sites which seem to be unrelated to transport, but the major fraction of MITC reactive sites (~300 000/cell) appear to be transport related in that transport was totally blocked when these were occupied. The 90 000 sites which appear to be transport independent may reside at positions on the monosaccharide transporter which are not essential for sugar transport; alternatively, since MITC is a reactive isothiocyanate, its reaction with these sites may simply represent nonspecific reaction with other band 3 components such as the anion transporter. Further work will be necessary to distinguish between these possibilities. In addition to its reaction with band 3, ~25 000 residues of [^{14}C]MITC per cell were incorporated into band 4.5. However, this incorporation was unchanged by the presence of substrates or competitive inhibitors of the monosaccharide transporter under conditions such that transport inhibition and labeling of band 3 by MITC were both substantially antagonized. By this criterion, band 4.5 appears to be unrelated to glucose transport in intact erythrocytes prepared from freshly drawn blood.

On the other hand, labeled band 3 in intact erythrocytes could be converted to labeled band 4.5 by externally added chymotrypsin or by an endogenous process which was inhibited by phenylmethanesulfonyl fluoride and was presumably proteolytic. Since we showed that this endogenous conversion was also rather extensive in neutral detergent extracts of the labeled erythrocyte membrane, and since the red cell membrane is known to contain proteases which are activated by neutral detergents (Tokes & Chambers, 1975), it seems possible that those investigators who have found that band 4.5 was the major component of detergent-solubilized membranes which was active in reconstituting transport had inadvertently converted the glucose transporter of band 3 to band 4.5 by the action of endogenous protease attack. Such a fragment might be expected to have glucose transport activity since it has been found that external digestion of erythrocyte ghosts with trypsin resulted in conversion of band 3 to components migrating in the band 4.5 region without diminishing glucose transport (Avruch et al., 1973). Another possibly significant difference between our experiments and those of other investigators who have concluded that band 4.5 is the transporter is that we have used only freshly drawn blood for our experiments. However, both those investigators who have carried out reconstitution experiments with detergent extracts (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Zoccoli et al., 1978) and those who have identified the glucose transporter as a band 4.5 component by the use of impermeant maleimides (Batt et al., 1976) have used outdated blood bank blood as a source of erythrocytes for their experiments. In view of our data which show that there is rather rapid degradation of MITC-labeled band 3 to labeled band 4.5 in incubated washed erythrocytes, it seems possible that a similar conversion may have occurred during storage of whole blood for 5–7 weeks in the blood bank. Obvious changes in erythrocyte membrane proteins have been observed under this circumstance (Moore et al., 1971). This conversion might have escaped detection, because we observed only a small change in distribution of the Coomassie Brilliant Blue stained proteins after NaDodSO$_4$–polyacrylamide gel electrophoresis of washed cells which had been incubated. This implies that the band 3 component which is labeled by MITC differs from the bulk of band 3 in its sensitivity to degradation and thus may be structurally different as well.

Band 3 is the major intrinsic protein component of the erythrocyte membrane which has been known for some time to span the membrane (Bretscher, 1971; Phillips & Morrison, 1971a,b; Mueller & Morrison, 1974). Each cell contains approximately 1.2 million copies of band 3 protein (Fairbanks et al., 1971). It is of considerable interest that the erythrocyte membrane transporters for glucose, anions (Cabantchik & Rothstein, 1974a,b; Rothstein et al., 1976; Lepke et al., 1976; Ship et al., 1977; Rakitzis et al., 1978; Ross & McConnell, 1977; Ho & Guidotti, 1975), and cations (Avruch & Fairbanks, 1972) are all components of band 3. Furthermore, evidence exists which suggests that there are approximately 300 000 copies of each transporter per cell (Avruch & Fairbanks, 1972; Ho & Guidotti, 1975), although the number of anion transporters is unsettled (Rakitzis et al., 1978). In addition, it has been reported that external proteolysis under conditions which lead to conversion of band 3 to fragments of approximately 55 000 and 40 000 molecular weight does not inhibit the functions of the transporters for glucose (Avruch et al., 1973; Carter et al., 1973; Kahlenberg et al., 1972), cations (Avruch & Fairbanks, 1972; Wagner et al., 1974), or anions (Rothstein et al., 1976). Despite these similarities, in intact cells each transport function appears to be independent of the others. As we have shown, prior treatment of erythrocytes with DIDS or 4-sulfophenyl isothiocyanate under conditions which lead to complete inactivation of anion transport had little effect on either glucose transport or MITC incorporation. Furthermore, there is no apparent direct relationship between glucose and sodium transport in erythrocytes. It is apparent that those observations which suggest that the transporters are functionally distinct must be reconciled with topographical studies which have been interpreted to mean that all band 3 molecules are structurally identical (Steck et al., 1978; Drickamer, 1978; Fukuda et al., 1978; Reithmeier, 1979). If this hypothesis were correct, it would be necessary to account for the obvious functional differentiation of this protein on some basis other than its primary structure. On the other hand, amino acid sequence data for this protein are so limited that it may be premature to conclude that all band 3 molecules are structurally identical. Indeed, our data which demonstrate that the glucose transporter is more susceptible to endogenous proteolysis than the remainder of band 3 strongly suggest that it is structurally different as

well as functionally distinct from other components. It seems possible that band 3 may be composed of a family of transmembrane transport proteins which are closely related structurally, but whose structures have evolved to accommodate specific transport functions.

References

Abbot, R. E., & Schachter, D. (1976) *J. Biol. Chem. 251*, 7176.

Avruch, J., & Fairbanks, G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1216.

Avruch, J., Price, H. D., Martin, D. B., & Carter, J. R. (1973) *Biochim. Biophys. Acta 291*, 494.

Batt, E. R., Abbot, R. E., & Schachter, D. (1976) *J. Biol. Chem. 251*, 7184.

Bernacki, R. J., & Bosmann, H. B. (1972) *J. Membr. Biol. 7*, 1.

Bretscher, M. S. (1971) *J. Mol. Biol. 59*, 351.

Cabantchik, Z. I., & Rothstein, A. (1974a) *J. Membr. Biol. 15*, 207.

Cabantchik, Z. I., & Rothstein, A. (1974b) *J. Membr. Biol. 15*, 227.

Carter, J. R., Jr., Avruch, J., & Martin, D. B. (1973) *Biochim. Biophys. Acta 291*, 506.

Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119.

Drickamer, L. K. (1978) *J. Biol. Chem. 253*, 7242.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Fukuda, M., Eshdat, Y., Tarone, G., & Marchesi, V. T. (1978) *J. Biol. Chem. 253*, 2419.

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

Groman, E. F., Schultz, R. M., & Engel, L. L. (1977) *Methods Enzymol. 46*, 54.

Ho, M. K., & Guidotti, G. (1975) *J. Biol. Chem. 250*, 675.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 304.

Jones, M. N., & Nickson, J. K. (1978) *Biochim. Biophys. Acta 509*, 260.

Jung, C. Y., & Carlson, L. M. (1975) *J. Biol. Chem. 250*, 3217.

Jung, C. Y., & Rampal, A. L. (1977) *J. Biol. Chem. 252*, 5456.

Kahlenberg, A. (1976) *J. Biol. Chem. 251*, 1582.

Kahlenberg, A., & Zala, C. A. (1977) *J. Supramol. Struct., Suppl. 1*, 149.

Kahlenberg, A., Urman, B., & Dolansky, D. (1971) *Biochemistry 10*, 3154.

Kahlenberg, A., Dolansky, D., & Rohrlick, R. (1972) *J. Biol. Chem. 247*, 4572.

Kasahara, M., & Hinkle, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 396.

Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lepke, S., Fasold, H., Pring, M., & Passow, H. (1976) *J. Membr. Biol. 29*, 147.

Lienhard, G. E., Gorga, F. R., Orasky, J. E., Jr., & Zoccoli, M. A. (1977) *Biochemistry 16*, 4921.

Lin, D. C., & Lin, S. (1978) *J. Biol. Chem. 253*, 1415.

Lin, S., & Spudich, J. A. (1974) *J. Biol. Chem. 249*, 5778.

Lin, S., & Snyder, C. E., Jr. (1977) *J. Biol. Chem. 252*, 5464.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Moore, G. L., Cooper, D. A., Antonoff, R. S., & Robinson, S. L. (1971) *Vox Sang. 20*, 239.

Mueller, T. J., & Morrison, M. (1974) *J. Biol. Chem. 249*, 7568.

Mullins, R. E., & Langdon, R. G. (1980) *Biochemistry* (preceding paper in this issue).

Phillips, D. R., & Morrison, M. (1971a) *Biochem. Biophys. Res. Commun. 45*, 1103.

Phillips, D. R., & Morrosion, M. (1971b) *FEBS Lett. 18*, 95.

Phutrakul, S., & Jones, M. N. (1979) *Biochim. Biophys. Acta 550*, 188.

Pinkofsky, H. B., Rampal, A. L., Cowden, M. A., & Jung, C. Y. (1978) *J. Biol. Chem. 253*, 4930.

Rakitzis, E. T., Gilligan, P. J., & Hoffman, J. F. (1978) *J. Membr. Biol. 41*, 101.

Reithmeier, R. A. F. (1979) *J. Biol. Chem. 254*, 3054.

Ross, A. H., & McConnell, H. M. (1977) *Biochem. Biophys. Res. Commun. 74*, 1318.

Rothstein, A., Cabantchik, Z. I., & Knauf, P. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 3.

Ship, S., Shami, Y., Breuer, W., & Rothstein, A. (1977) *J. Membr. Biol. 33*, 311.

Steck, T. L., Koziarz, J. J., Singh, M. K., Reddy, G., & Kohler, H. (1978) *Biochemistry 17*, 1216.

Taverna, R. D., & Langdon, R. G. (1973a) *Biochim. Biophys. Acta 323*, 207.

Taverna, R. D., & Langdon, R. G. (1973b) *Biochem. Biophys. Res. Commun. 54*, 593.

Tokes, Z. A., & Chambers, S. M. (1975) *Biochim. Biophys. Acta 389*, 325.

Trosper, T., & Levy, D. (1977) *J. Biol. Chem. 252*, 181.

Wagner, H., Jr., Smith, T. W., & Young, M. (1974) *Arch. Biochem. Biophys. 163*, 95.

Zala, C. A., & Kahlenberg, A. (1976) *Biochem. Biophys. Res. Commun. 72*, 866.

Zoccoli, M. A., & Lienhard, G. E. (1977) *Biochem. Biophys. Res. Commun. 76*, 446.

Zoccoli, M. A., Baldwin, S. A., & Lienhard, G. E. (1978) *J. Biol. Chem. 253*, 6923.