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Monosaccharide Transport System of the Human Erythrocyte. Identification of the Cytochalasin B Binding Component†

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ABSTRACT: Sealed, protein-depleted vesicles were formed by treatment of erythrocyte membranes with dimethylmaleic anhydride and isolated by density gradient centrifugation. These vesicles contain the major erythrocyte membrane polypeptides of bands 3, 4.5, 7, and PAS 1-3 but lack those of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 (nomenclature of Steck, T. L. (1974), *J. Cell. Biol. 62,* 1). The presence of the monosaccharide transport system in the vesicles was demonstrated by the findings that the vesicles transport the substrate L-sorbose and that this uptake is inhibited by cytochalasin B, phloretin, and D-glucose. The activity of the transport system (initial rate of L-sorbose uptake per minute per milligram of membrane protein) is about 95% of that in intact cells. The transport of L-sorbose and the binding of [^{3}H]cytochalasin B to the vesicles are inactivated by reaction of the vesicles with 1-fluoro-2,4-dinitrobenzene. Cytochalasin B protects these functions against inactivation. Differential labeling of the cytochalasin B binding component was accomplished by first treating the vesicles with nonradioactive 1-fluoro-2,4-dinitrobenzene in the absence and presence of cytochalasin B, and then reacting them in the absence of cytochalasin B with tritium-labeled reagent in one case and with ^{14}C-labeled reagent in the other case. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the combined treated vesicles showed a region of selective labeling that coincided with protein band 4.5 (molecular weight range 45 000-65 000). The number of dinitrophenyl groups that can be differentially incorporated, after correction for incomplete protection and partial reaction, is equivalent to about 350 000 per cell. This value agrees approximately with the number of high affinity binding sites for cytochalasin B in the erythrocyte membrane.

The monosaccharide transport system of the human erythrocyte has been extensively investigated with regard to its kinetics, specificity, and susceptibility to inhibition (LeFevre, 1961; LeFevre, 1975; Jung, 1975). Efforts are now underway in a number of laboratories to identify and isolate the components of this transport system (please see the Discussion). Here we report the results of an approach to this problem that relies on the following earlier findings. First, treatment of the erythrocyte membrane with dimethylmaleic anhydride releases most of the peripheral membrane proteins (Steck and Yu, 1973). Second, the erythrocyte membrane and the membrane depleted of peripheral proteins by reaction with dimethylmaleic anhydride possess a set of high-affinity binding sites for cytochalasin B that appear to be a component of the monosaccharide transport system (Lin and Spudich, 1974a,b). Third, N_2ph-F[1] inactivates the transport system in intact cells (Bowyer and Widdas, 1958; Bloch, 1974) and resealed erythrocyte membranes (Jung, 1974), and cytochalasin B protects against this inactivation (Bloch, 1973).

Our rationale has been that identification of the cytochalasin B binding component through differential labeling with N_2ph-F would have the best chance of success if a membrane preparation with a simplified protein composition were used. To this end, we have treated human erythrocyte membranes with dimethylmaleic anhydride to obtain vesicles that lack the peripheral membrane proteins. The monosaccharide transport system was found to be functional in these vesicles. It is inactivated by N_2ph-F, and cytochalasin B protects against this inactivation. These properties have allowed us to identify the cytochalasin B binding component of the transport system through differential labeling with N_2ph-F.

Experimental Procedures

Materials. L-[^{14}C]Sorbose, [^{14}C]N_2ph-F, and [^{3}H]N_2ph-F were purchased from Amersham-Searle. [^{3}H]Cytochalasin B, obtained from New England Nuclear, was purified as described previously (Lienhard and Wardzala, 1976). Freshly outdated units of whole blood or packed cells in citrate-phos-

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[1] Abbreviations used are: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; PAS, periodic acid-Schiff reagent.

phate-dextrose were kindly provided by the blood bank of the Mary Hitchcock Memorial Hospital.

Preparation of Protein-Depleted Vesicles. Erythrocyte membranes were prepared from washed cells according to the procedure of Steck and Kant (1974). To 300 mL of membranes, at a protein concentration of about 0.70 mg/mL, in 1.25 mM sodium phosphate (pH 8) was added enough solid dimethylmaleic anhydride to give 15 mM. The pH was maintained at 8 ± 0.3 by addition of 0.25 N NaOH. After the formation of acid had ceased (about 1 h at room temperature), the membrane fragments were isolated by centrifugation at 36 000*g* for 15 min. All further operations were performed at 4 °C. The membrane fragments were suspended in 360 mL of 1 mM sodium phosphate (pH 8), sedimented by centrifugation, and then resuspended in about 9 mL of the same buffer. The suspension was gently homogenized with two strokes of the pestle in a glass homogenizer with Teflon pestle (Arthur H. Thomas 3431-E15) and then layered on linear density gradients of Dextran T70 in 0.5 mM sodium phosphate (pH 8) that varied in density from 1.00 to 1.04 g/cm^3. Centrifugation was carried out at 37 000 rpm for 75 min with a Beckman SW41 rotor. Generally, three bands were obtained, and these had average densities of about 1.002, 1.010, and 1.027 g/cm^3. In an occasional preparation, the lighter two bands were replaced by a somewhat wider single band. Measurements of L-sorbose uptake revealed that both lighter fractions consisted of sealed vesicles, whereas the heavy band consisted of unsealed membrane fragments. The lighter bands were each collected in about 10 mL and diluted to 40 mL with 5 mM sodium phosphate (pH 8). The vesicles were sedimented at 36 000*g* for 15 min, resuspended in 40 mL of 5 mM sodium phosphate (pH 8), and sedimented again. Finally, they were resuspended at a protein concentration of 2.0 mg/mL with thorough mixing in order to disperse wisps of clumped vesicles. The vesicles were stored in the refrigerator and used within 3 days. A typical preparation yielded about 12 mg of protein in the lightest fraction and 8 mg of protein in the intermediate fraction, from about 200 mg of membrane protein.

The two fractions of sealed vesicles had the same polypeptide composition and membrane orientation (see Results). The initial rate of L-sorbose uptake by the lighter fraction was about 50% greater than that of the other sealed fraction. Also, the average internal volume of the lighter fraction, calculated on the basis of the amount of L-sorbose uptake at equilibrium, was about 50% greater. For most experiments, we have used a mixture of both vesicle populations in the proportions isolated.

Uptake of L-Sorbose. The rates of entry of L-[^{14}C]sorbose into the vesicles were measured at 12.5 °C in 5 mM sodium phosphate (pH 7.5 or 8) by a filtration procedure that is described in detail elsewhere (Zoccoli and Lienhard, 1977). When cytochalasin B was included in the uptake mixtures, it was added as an aliquot of a concentrated solution in ethanol such that the ethanol was less than 2.5% (v/v); the corresponding control mixture received an equal volume of ethanol. The initial rate of uptake was not altered by 2.5% ethanol alone.

Binding of Cytochalasin B to the Vesicles. [^{3}H]Cytochalasin B (5 μL of 10^{-6} M in 10% ethanol) was mixed with 500 μL of vesicles (100 μg of protein) in 5 mM sodium phosphate (pH 7.5), in a 1-mL conical glass centrifuge tube. A 50-μL aliquot was taken to determine the total concentration of cytochalasin B by measurement of radioactivity. After 15 min at room temperature, the mixture was centrifuged at 15 000 rpm in a Sorvall SS34 rotor. On the basis of protein assay, over 95% of the vesicles sedimented in the pellet. A 50-μL aliquot of the supernatant was removed to determine the concentration of unbound cytochalasin B.

These data were used to estimate the relative concentration of high-affinity cytochalasin B sites in the following way. The dissociation constant (K_d) is expressed by:

$$K_d = [R][L]/[RL]$$

where [R] and [RL] are the concentrations of unoccupied and occupied high-affinity cytochalasin B binding sites, respectively, and [L] is the concentration of unbound cytochalasin B. The value of K_d is 10^{-7} M (Lin and Spudich, 1974a,b); hence, with a total concentration of 10^{-8} M cytochalasin B, the ratio of [R]/[RL] must be 10 or more. Consequently, the total concentration of sites ($[R]_t$) is approximately equal to [R], and thus $[R]_t$ approximately equals $K_d[RL]/[L]$. Therefore, we have used the ratio of bound to unbound cytochalasin B as a measure of the relative concentration of sites (Figure 3 and Table I).

Reaction of Vesicles with Radioactive N_2ph-F. [^{3}H]N_2ph-F (200 mC/mmol) is supplied as a 5 mM solution in benzene. [^{14}C]N_2ph-F (21 mCi/mmol) was dissolved in enough benzene to give 5 mM. Aliquots (50 μL) (0.25 μmol) were placed in 12 × 75 mm glass tubes and allowed to evaporate for 17 h in the hood. A control experiment with [^{14}C]benzene and nonradioactive N_2ph-F showed that less than 0.01 μmol of benzene remained after this period, whereas the amount of N_2ph-F that remained, determined spectrophotometrically by alkaline hydrolysis to dinitrophenolate (Bunton and Robinson, 1969), was 0.22 μmol. To each residue of radioactive N_2ph-F was added 100 μL of vesicles at a protein concentration of 2 mg/mL in 5 mM sodium phosphate (pH 7.5)–2.5% ethanol that previously had been reacted with nonradioactive 2 mM N_2ph-F in the presence or absence of 10 μM cytochalasin B for 5 h and then washed extensively. The procedure for the initial treatment with N_2ph-F is described in detail in Table I of the Results. The reaction mixtures were mixed well to ensure dissolution of the N_2ph-F and left at 25.0 °C for 4 h. After this period, they were diluted to 1 mL with 5 mM sodium phosphate (pH 7.5)–2.5% ethanol. Then individual reaction mixtures were combined in pairs as described in Table II of the Results, diluted to 10 mL with the same buffer, and filtered in 5-mL amounts through 25-mm HAWP Millipore filters (2 filters per combination). Each filter was washed ten times with 2-mL aliquots of the buffer. Each pair of filters was shaken for 30 min with 500 μL of 5 mM *N*-ethylmaleimide–1 mM EDTA–10 mM Tris-Cl–1% sodium dodecyl sulfate (pH 8). The extracts were concentrated to 100 μL under an N_2 stream at 50 °C; 10 μL of 84% sucrose and 5 μl of 12 mg/mL Pyronin Y were added to each. Aliquots of 29 μL were subjected to polyacrylamide gel electrophoresis. The gels were shaken for 12-h periods with 50 mL of the following: 25% 2-propanol–10% acetic acid (3 times), 10% acetic acid, 1% acetic acid. They were then frozen and sliced into 2-mm sections. Each slice was digested by shaking for 24 h at 30 °C with 10 mL of scintillation cocktail (92 mL of Amersham–Searle NCS tissue solubilizer, 9 mL of 4 M NH_4OH, 4.2 g of 2,5-diphenyloxazole, and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 L of toluene). Radioactivity was determined with a Packard Model 3385 liquid scintillation spectrometer set so that there was less than 0.005% spillover of tritium into the ^{14}C channel.

Other Procedures. Vesicles were examined with a Zeiss Axiomat microscope equipped with high extinction Nomarski differential interference contrast optics and an ocular micrometer, which is on loan from the Zeiss Company to Dr. Robert D. Allen of the Department of Biological Sciences, Dartmouth College. We are indebted to Jeffrey L. Travis and

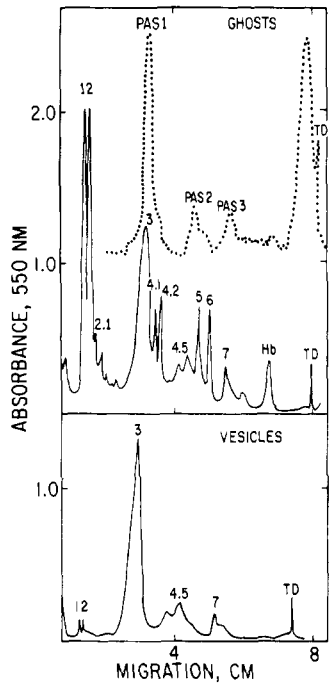

FIGURE 1: Polypeptide composition of erythrocyte membranes and protein-depleted vesicles. Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Fairbanks et al. (1971), with the exception that they were held at 100 °C for 4 min rather than 37 °C for 15 min. In this method, dithiothreitol is included to reduce the protein disulfide bonds. The solid lines are scans of gels to which about 10 μg of protein had been applied and which were stained with Coomassie blue. The dotted line is the scan of a gel to which about 30 μg of protein had been applied and which was stained with periodic acid-Schiff (PAS) reagent. In this latter case, scanning was done at 560 nm, and the height of PAS 1 is about 0.36 absorbance unit. The polypeptide bands are designated according to the nomenclature of Steck (1974). TD marks the position of the tracking dye. The pattern of staining with periodic acid-Schiff reagent given by the vesicles was identical with that shown for the erythrocyte membranes.

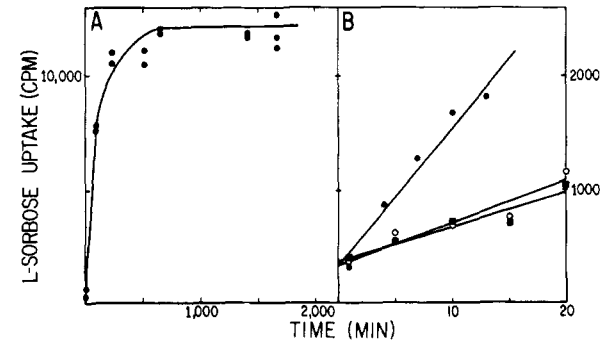

FIGURE 2: Rates of L-[^{14}C]sorbose uptake by vesicles. The method is described in the Experimental Procedures. The uptake values are those for 50-μL aliquots of the assay mixture. The total radioactivity in these aliquots was about 200 000 cpm. Part A shows the uptake to equilibrium, and part B shows the initial rate of uptake in the presence of no (●), 25 (○), and 50 (■) μM cytochalasin B. The lines in part B are the best fit according to linear least-squares analysis.

Dr. Allen for this examination. The determination of membrane orientation by assays of acetylcholinesterase was performed by the method of Steck and Kant (1974). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as well as the subsequent staining with Coomassie blue or periodic acid-Schiff reagent, were carried out as described by Steck and Yu (1973). Protein was determined by the Lowry method with crystalline bovine serum albumin as the standard (Lowry et al., 1951).

Results

Properties of Protein-Depleted Vesicles. Protein-depleted vesicles were prepared by reacting erythrocyte membranes with dimethylmaleic anhydride and isolating the sealed vesicles through density gradient centrifugation (see the Experimental Procedures). The sizes of the vesicles, which were estimated by light microscopy, ranged from about 0.50 to 1.50 μm. The orientation of the vesicular membrane, relative to that of the intact cell, was evaluated by measurement of the accessibility of the enzyme acetylcholinesterase (Steck and Kant, 1974). The average value from 25 preparations was 75% inside-out, with a range from 43 to 87% inside-out. The polypeptide composition of the depleted vesicles is compared with that of the erythrocyte membrane in Figure 1. In agreement with the expectation from the results of Steck and Yu (1973), the vesicles contain predominantly bands 3, 4.5, 7, and PAS 1-3; little or none of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 remain.

Monosaccharide Transport by the Protein-Depleted Vesicles. L-Sorbose is a substrate with kinetic parameters convenient for the assay of the monosaccharide transport system in vesicles (Zoccoli and Lienhard, 1977). The time course for uptake of L-sorbose to equilibrium and the initial rate of uptake are illustrated in Figure 2. Cytochalasin B is a potent inhibitor of the monosaccharide transport system in intact erythrocytes. The finding that 25 and 50 μM cytochalasin B each inhibit the initial rate of entry by about 75% (Figure 2) is evidence that this fraction of the uptake proceeds by way of the transport system. Since these concentrations of cytochalasin B are enough to inhibit the system in erythrocytes by over 98% (Taverna and Langdon, 1973; Bloch, 1973), the 25% of the initial rate that is not inhibited is probably due to entry by other pathways. We have also found that under the conditions described in Figure 2, the initial rate of L-sorbose uptake is inhibited 50% by 50 mM D-glucose and 69% by 40 μM phloretin. When correction is made for the 25% entry that is not inhibitable by cytochalasin B, the magnitudes of these inhibitions are consistent with the effects of these compounds upon transport in intact cells (Zoccoli and Lienhard, 1977) and provide further evidence that a large fraction of the transport occurs by way of the monosaccharide transport system.

In the presence of 0.5 mM L-sorbose at 12.5 °C and pH 8, the values of the initial rates of entry into 19 preparations of protein-depleted vesicles averaged 175 pmol min^{-1} (mg of protein)$^{-1}$, with a range from 130 to 245. Under the same conditions, the value for intact erythrocytes is 140 pmol min^{-1} (mg of membrane protein)$^{-1}$.[2] Consequently, after correcting for the 25% of entry that occurs by other routes, we find that the activity of the transport system in the vesicles is about 95% of that in intact cells.

N_2ph-F Inactivation of Transport and Cytochalasin B Binding. Treatment of the protein-depleted vesicles with N_2ph-F resulted in inactivation of both monosaccharide transport and cytochalasin B binding. The time courses for loss of activity are shown in Figure 3. The loss of transport activity is preceded by a lag of about 1 h (see Discussion), whereas the loss of cytochalasin B binding does not show such a lag. Both activities disappear with a half-time of about 1.5 h and reach low values by 6 h.

The inactivation by N_2ph-F can be prevented by cytochalasin B. Table I summarizes the results from two protection experiments. In the presence of 10 μM cytochalasin B, the recovered transport and binding activities averaged 77 and 93% of the control values, respectively, while in its absence these fall to average values of 17 and 8%.

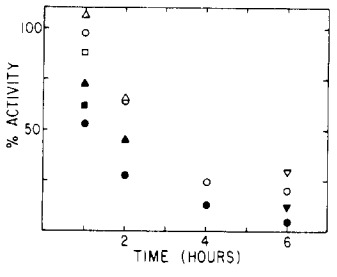

FIGURE 3: Time courses for N_2ph-F inactivation of L-sorbose uptake by the monosaccharide transport system and of cytochalasin B binding. Vesicles, at 2 mg of protein/mL in 5 mM sodium phosphate (pH 7.50) -2.5% ethanol, were reacted with 2 mM N_2ph-F at 25.0 °C. At each time, a 1- to 3.5-ml aliquot was removed, diluted with 40 mL of ice-cold 5 mM sodium phosphate (pH 7.5), and centrifuged at 20 000 rpm in a Sorvall SS34 rotor for 10 min. The pelleted vesicles were resuspended in another 40 mL of ice-cold buffer, centrifuged again, and finally resuspended at a protein concentration of 2 mg/mL. The initial rates of L-sorbose uptake in the absence and presence of 25 μM cytochalasin B were then determined. The activity of the transport system was taken to be the difference between these two initial rates. [^{3}H]Cytochalasin B binding was also measured. The results are expressed as percentages of the values for a sample taken just before the addition of N_2ph-F (zero time). Open symbols show the percentage of transport activity that remains; closed ones, that of binding activity. A single shape of symbol is used for the results obtained in each experiment. In the absence of N_2ph-F, there was no change in either activity over a period of 8 h.

TABLE I: Protection by Cytochalasin B against N_2ph-F Inactivation of L-Sorbose Uptake and Cytochalasin B Binding.[a]

	Treatment	Rate of uptake (pmol min^{-1} mg^{-1}) −CytoB	+CytoB	% transport act.[b]	[CytoB] bound / [CytoB] free	% binding act.
A.	None	129	39	100	2.40	100
	N_2ph-F	89	63	29	0.28	12
	CytoB + N_2ph-F	151	75	85	2.66	111
B.	None	180	24	100	2.33	100
	N_2ph-F	71	63	5	0.09	4
	CytoB + N_2ph-F	181	75	69	1.78	76

[a] To 2 mL of vesicles in 5.5 mM sodium phosphate (pH 7.50) the following were added: 50 μL of ethanol (none), 50 μl of 80 mM N_2ph-F in ethanol (N_2ph-F), or 10 μL of 2 mM cytochalasin B in ethanol followed by 40 μL of 100 mM N_2ph-F in ethanol (CytoB + N_2ph-F). The vesicles were maintained at 25.0 °C for 5 (experiment A) or 6 (experiment B) h. After 2.5 h, 5 μL of 100 mM NaOH was added; this addition raised the pH in the N_2ph-F-containing mixtures from 7.45 to 7.50. At the end of the reaction period, the mixtures were diluted with 38 mL of 5 mM sodium phosphate (pH 7.5), and the vesicles were separated by centrifugation at 20 000 rpm in a Sorvall SS34 rotor for 10 min. The vesicles were resuspended in 40 mL of the same buffer and separated by centrifugation again. This washing procedure was repeated 3 more times. Then the initial rates of L-sorbose uptake in the absence (−CytoB) and presence (+CytoB) of 25 μM cytochalasin B and the relative concentration of cytochalasin B binding sites ([CytoB] bound/[CytoB] free) were assayed as described in the Experimental Procedures. [b] The transport activity was taken to be the difference between the −CytoB and +CytoB values.

TABLE II: Protocol for Selective Labeling of a Cytochalasin B Binding Protein.[a]

Initial treatment	Labeling treatment	Combinations for electrophoresis
1. 2 mM N_2ph-F	A. [^{14}C] N_2ph-F	
	B. [^{3}H]N_2ph-F	1A + 2B
		1A + 1B
2. 10 μM cytochalasin B and 2 mM N_2ph-F	A. [^{14}C] N_2ph-F	1B + 2A
	B. [^{3}H] N_2ph-F	

[a] See the Experimental Procedures for details.

Differential Labeling of the Cytochalasin B Binding Protein. In order to reduce the amount of nonspecific incorporation of radioactive dinitrophenyl residues, the following protocol was adopted (Table II). Some vesicles were first reacted with nonradioactive N_2ph-F in the absence of cytochalasin B; by this treatment many groups, including the one that is protected by cytochalasin B, undergo dinitrophenylation. Other vesicles were first reacted with nonradioactive N_2ph-F in the presence of cytochalasin B; by this treatment the same groups, with the exception of the cytochalasin B protected site, react. After removal of the nonradioactive N_2ph-F and, in the second case, the cytochalasin B, each vesicle population was divided into two parts, one of which was treated with [^{14}C]N_2phF and the other with [^{3}H]N_2phF. The vesicles were then combined in such a way that selective labeling of the polypeptide that is protected by cytochalasin B from reaction with the nonradioactive N_2phF would lead to an increase in the ratio of tritium to ^{14}C relative to that of other proteins (1A + 2B, Table II), a decrease in this ratio (1B + 2A, Table II), or no change in the ratio (1A + 1B, Table II). The results are illustrated in Figure 4. All the regions of the gel containing major polypeptide components on the basis of staining (bands $[3]_2$, 3 and PAS 1, 4.5 and PAS 2, 7 and PAS 3), as well as the lipid, show peaks of radioactivity (lower section). The expected ratio shifts for differential labeling occur only in the band 4.5 region (middle section). The excess amount of ^{14}C or tritium in each slice in this region as the result of the selective labeling was estimated as follows. The amount of isotope expected if there were no selective labeling was calculated from the average ratio of tritium to ^{14}C in the other protein bands and the amount of radioactivity in the slice due to the isotopic reagent applied to the vesicles that had been unprotected in the prior treatment. This value was then subtracted from the observed value. There is a peak of excess radioactivity for both tritium and ^{14}C that corresponds closely to the peak of Coomassie blue staining and total radioactive labeling for band 4.5 (top section). A repetition of this entire labeling experiment gave identical results. From the total amounts of excess isotope incorporated and the specific activities of the reagent, we calculate that 1.1 to 1.2 nmol of dinitrophenyl groups per mg of protein was selectively incorporated into the polypeptides of band 4.5.

Discussion

In a recent paper we described the preparation, from human erythrocyte membranes, of vesicles that contain little or none of the polypeptides of bands 1, 2, 5, and 6, through treatment of the membranes with dilute alkali (Zoccoli and Lienhard, 1977). The activity of the transport system in these vesicles, expressed per milligram of membrane protein, was about 85% of that for intact erythrocytes.[2] The maximal possible amounts of bands 1, 2, 5, and 6 that remained were estimated from scans

[2] In an earlier paper (Zoccoli and Lienhard, 1977) we described the determination of the initial rate for intact cells. When expressed in the above units (picomoles minute^{-1} (mg of membrane protein, according to the Lowry assay)$^{-1}$), the value reported therein is 200. We have recently redetermined this initial rate and, in the process, discovered that this earlier value is incorrect due to a computational error. The membrane protein content of a single erythrocyte, as determined by the Lowry assay, is 1.0×10^{-9} mg (Zoccoli and Lienhard, 1977).

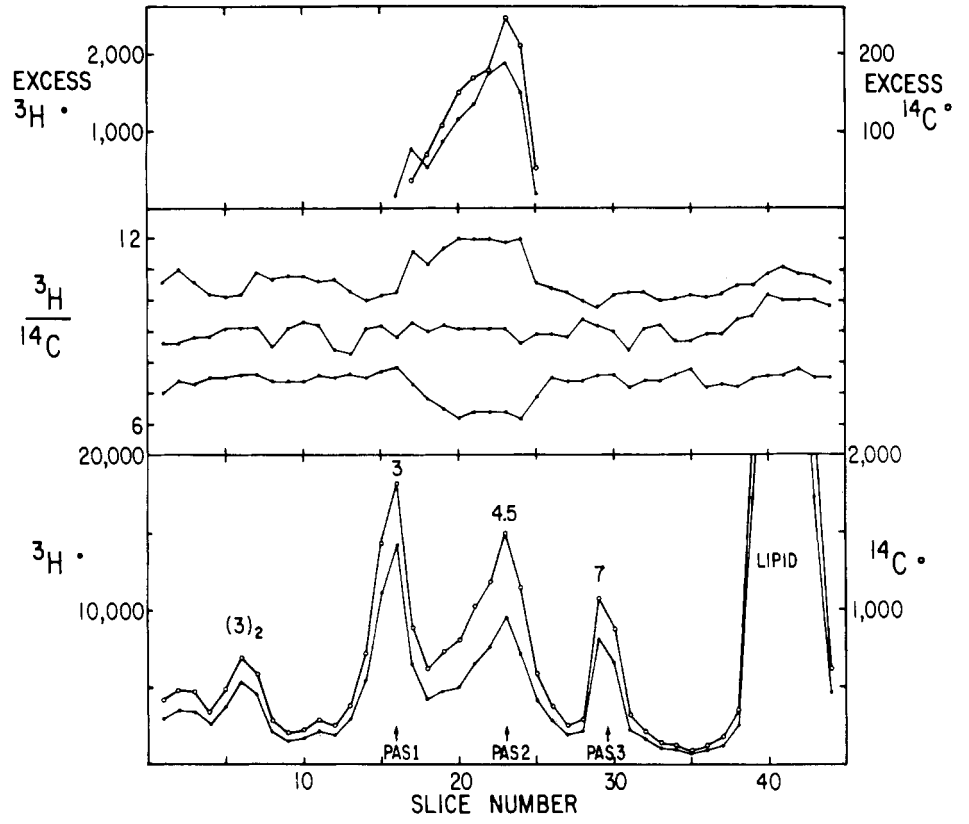

FIGURE 4: Differential labeling of a cytochalasin B binding protein. The pattern of incorporation of ^3H- and ^{14}C-labeled dinitrophenyl groups is illustrated in the lower panel with the data for the mixture 1B + 2A described in Table II. The location of the polypeptides was determined by staining of other gels run at the same time. The patterns of Coomassie blue and periodic acid–Schiff reagent staining were identical with those in Figure 1, with the exception that approximately one-fourth of the band 3 polypeptide was present as the disulfide-linked dimer $(3)_2$ (Steck, 1972) because the polypeptides were not reduced. The ratios of $^3H/^{14}C$ in the middle panel are those for mixtures 1A + 2B (top curve), 1A + 1B (middle curve), and 1B + 2A (lower curve) described in Table II. The raw values of the ratios for the 1A + 1B mixture have been multiplied by a factor of 1.1 to facilitate presentation. The upper panel gives the values of excess ^3H for the mixture 1A + 2B and excess ^{14}C for the mixture 1B + 2A, the computations of which are described in the text. The units of radioactivity for the upper and lower panel are counts per minute. See the Experimental Procedures for other details.

of polyacrylamide gels. It was shown that these amounts were insufficient to account for the recovered transport activity, and consequently concluded that the major polypeptides of these bands do not have an obligatory role in transport. Our present results with the protein-depleted vesicles obtained by treatment of erythrocyte membranes with dimethylmaleic anhydride are similar. The average value of the transport activity of the vesicles is 95% of that for intact cells. An analysis of gel scans (Figure 1) to estimate the maximal residual amounts of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 (data not given; seeZoccoli and Lienhard, 1977, for the method) shows that there is not enough of the major polypeptides of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 to account for the recovery activity.

Cytochalasin B at low concentration (10 μM) protects a group on one (or possibly more than one) polypeptide species in the band 4.5 region from reaction with N_2ph-F. This protection allows the subsequent differential labeling of the polypeptide (Figure 4). For several reasons, it is very likely that this polypeptide is the high-affinity receptor for cytochalasin B in the erythrocyte membrane. First, N_2ph-F inactivates the high-affinity binding of cytochalasin B to the vesicles (Figure 3), and cytochalasin B protects against the inactivation (Table I). Second, an estimate of the number of sites that can differentially incorporate dinitrophenyl groups agrees with the number of high-affinity binding sites for cytochalasin B on the erythrocyte membrane. In four experiments, 1.1 to 1.2 nmol of dinitrophenyl groups per mg of protein was differentially incorporated. Since prior treatment with nonradioactive N_2ph-F in the presence of cytochalasin B resulted in the recovery of 93% of the cytochalasin B binding activity (Table I) and since treatment with radioactive reagent should lead to a loss of 87% of the cytochalasin B binding activity (Figure 3), there are about 1.4 nmol of sites per mg of protein. About 60% of the protein of the erythrocyte membrane is released by the treatment with dimethylmaleic anhydride. Consequently, there would be 0.55 nmol of sites per mg of erythrocyte membrane protein, which, on the basis of the membrane protein content per cell (Zoccoli and Lienhard, 1977), corresponds to about 350 000 sites per cell. Lin and Spudich (1974a) report that there are approximately 300 000 high-affinity sites for cytochalasin B per erythrocyte membrane. There is no loss of sites upon treating membranes with dimethylmaleic anhydride (Lin and Spudich, 1974b).

There is considerable evidence that most of the high-affinity sites for cytochalasin B in the erythrocyte membrane, and also in the membrane depleted of peripheral proteins by reaction with dimethylmaleic anhydride, are located on a component of the monosaccharide transport system (Lin and Spudich, 1974a,b; Jung and Rampal, 1976; Lin et al., 1977). The strongest point is that most of the cytochalasin B bound to high-affinity sites is displaced by monosaccharides that are good substrates for the transport system, such as D-glucose, but not by other monosaccharides, such as L-glucose. Some of our results with the vesicles are consistent with this conclusion in a straightforward way. Cytochalasin B inhibits transport (Figure 2), and it protects against the inactivation of the

transport system by N_2ph-F (Table I). On the other hand, in an uncomplicated case, one would expect that the rates of N_2ph-F inactivation of the transport system and of cytochalasin B binding would parallel each other. Yet, our data show a lag in the onset of N_2ph-F inactivation of the transport system that does not occur with the inactivation of binding (Figure 3). Since N_2ph-F inactivation of the transport system above pH 7 in intact erythrocytes (Kruppa, 1971; Bloch, 1974) and resealed membranes (Jung, 1974) follows pseudo-first-order kinetics without a lag, the vesicles exhibit an anomalous behavior. Further experimentation will be needed to elucidate the basis of this behavior and lack of coincidence between the time courses of the two inactivations.

In the protein-depleted vesicles, the band 4.5 region contributes about 20% of the Coomassie blue staining (Figure 1). The molecular weight range for band 4.5 is 45 000–65 000 (Fairbanks et al., 1971), and, thus, the amount of the cytochalasin B binding component (1.4 nmol/mg of protein) is about 8% of the protein. This difference in percentages suggests that band 4.5 contains several polypeptide components. One of these is the glycoprotein PAS 2 (Figure 4). This species is almost certainly not the differentially labeled protein, since PAS 1 is a noncovalently linked dimer of PAS 2 (Marton and Garvin, 1973; Furthmayr and Marchesi, 1976) and constitutes under our conditions of electrophoresis, by periodic acid–Schiff staining, 65% of the total of this glycoprotein; yet there is no differential labeling in the region of PAS 1 and band 3 (Figure 4).

The results reported here are in agreement with those of three other recent investigations aimed at the identification and isolation of protein components of the monosaccharide transport system. Batt et al. (1976) have identified a component that migrates in the band 4.5 region of polyacrylamide gels through the differential labeling of intact erythrocytes with an impermeant maleimide. Both cytochalasin B and D-glucose served as protecting agents. Kasahara and Hinkle (1977) and Kahlenberg and Zala (1977) have reported that optimal reconstitution of the erythrocyte monosaccharide transport system is achieved with a fraction that contains predominantly one or more polypeptide species of band 4.5. In contrast, a number of earlier labeling studies found differential incorporation into proteins of higher molecular weight. Jung and Carlson (1975) made use of the fact that D-glucose stimulates the inactivation of transport by N_2ph-F. They treated erythrocyte membranes with one radioisotope of N_2ph-F in the presence of sugar and the other radioisotope of N_2ph-F in its absence, and found selective labeling of a protein in the molecular weight range of 180 000. Shanahan and Jacquez (1976) carried out similar experiments, and selectively labeled a protein of molecular weight above 240 000. LeFevre et al. (1975) reacted cells and membranes with *N*-ethylmaleimide in the presence and absence of cytochalasin B and subsequently treated them with either tritiated or ^{14}C containing reagent. They also found that a protein of molecular 240 000 or greater was differentially modified. Taken together, these studies indicate that there may also be a high molecular weight protein (band 1 or 2?) that is involved in transport. Because the protein-depleted vesicles prepared by alkali (Zoccoli and Lienhard, 1977) or dimethylmaleic anhydride treatment (Figure 1) have lost almost all of the high molecular weight proteins, it seems likely that this larger component is not required for transport. Rather, it may interact at the cytoplasmic surface with the band 4.5 polypeptide, which we suspect to be the transmembrane component of the monosaccharide transport system.

Note Added in Proof

M. F. Shanahan and J. A. Jacquez report results that are qualitatively similar to those described here in an article to appear in a 1977 issue of *Membrane Biochemistry*.

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