High affinity cytochalasin B binding sites which are apparently unrelated to sugar transport have been found in human, bovine, and rabbit red blood cells. These sites are located on membrane proteins which are exposed to the cytoplasmic side of the cell membrane. They do not bind D-glucose, but bind cytochalasins A, B, C, D, E, and dihydrocytochalasin B. Unlike sugar transport-related cytochalasin B binding sites, these sites are not sensitive to p-chloromercuribenzoate, but are lost when red cell membranes are incubated at 37°C for 2 h, or extracted with dimethyl maleic anhydride or EDTA. The binding of cytochalasin B to these sites is rapid and reversible at 4°C, 23°C, and 37°C, and is enhanced by low pH and high ionic strength. Scatchard plot analysis of binding data obtained under equilibrium conditions shows that the dissociation constant of the cytochalasin B-receptor complex is $1 \times 10^{-8} \text{M}$ and that there are $4 \times 10^{4}$ sites per human or bovine red cell. These sites represent only a small fraction of the total number of high affinity cytochalasin B binding sites in the human red cell. However, they represent all of the high affinity sites present in bovine and rabbit red cells, which lack a sugar transport system similar to that found in human red cells. The biological function of this type of binding site in the red cell is not known. However, because they bind both cytochalasin B and dihydrocytochalasin B, compounds which affect cell motility and morphology, we postulate that these binding sites may be associated with contractile proteins.

Most of the biological effects of cytochalasin B on mammalian cells fall into one of two categories. At lower concentrations ($10^{-9}$ to $10^{-6} \text{M}$), the drug inhibits transport of small molecules (i.e. hexoses and nucleosides) across the cell membrane (Kletzen et al., 1972; Plagemann and Estensen, 1972). At higher concentrations ($10^{-6}$ to $10^{-4} \text{M}$), the drug affects cell motility and morphology (Wee et al., 1971).

We have previously reported that mammalian cells have two main classes of binding sites for cytochalasin B: high affinity sites with a dissociation constant ($K_d$) of $10^{-8} \text{M}$ and low affinity sites with a $K_d$ of $10^{-4} \text{M}$ (Lin et al., 1974). In the human red cell, the high affinity sites are associated with the cell membrane (ghost) (Lin and Spudich, 1974a). Because much of the binding of cytochalasin B to these sites can be inhibited by sugars which are substrates of the sugar transport system, we have concluded that most of these binding sites are intimately related to, if not identical with, the sugar transport proteins of the cell.

Because cytochalasin B affects mammalian cells in many different ways, it is likely that these cells contain different types of cytochalasin B binding sites. In *in vitro* experiments showed that cytochalasin B binds to actin with low affinity (Lin and Spudich, 1974b), leading to structural changes in this protein which can be detected by viscometry (Spudich and Lin, 1972) and electron microscopy (Spudich, 1972). Such interaction may lead to some of the effects on cell motility and morphology observed at $10^{-7}$ to $10^{-4} \text{M}$ cytochalasin B. On the other hand, many effects of cytochalasin B on cell motility and morphology can be observed at low drug concentrations ($10^{-8}$ to $10^{-6} \text{M}$). It is possible that high affinity cytochalasin B binding sites are involved in these phenomena. The latter conjecture is supported by recent reports that not all high affinity cytochalasin B binding sites are related to sugar transport (Jung and Rampal, 1976; Lin et al., 1977). This paper describes the properties of cytochalasin B binding sites located on red cell membrane proteins which are apparently unrelated to sugar transport. These sites differ from the sugar transport-related sites in that they (a) do not bind D-glucose, but bind cytochalasin B and dihydrocytochalasin B; (b) are found in nonprimate (i.e. bovine and rabbit) red cells, which lack a sugar transport system similar to that found in human red cells; and (c) are sensitive to dimethyl maleic anhydride and EDTA extraction and relatively insensitive to p-chloromercuribenzoate treatment.

EXPERIMENTAL PROCEDURES

**Materials** - Cytochalasins A, B, C, D, and E were purchased from Aldrich Chemical Co. [3H]Cytochalasin B (7.6 Ci/mmol) prepared by the method of Lin et al. (1974) was purchased from New England Nuclear. This material was comparable to that prepared in our laboratory, as judged by thin layer chromatography and high affinity binding to human red cell ghosts. Stock solutions of the cytochalasins were prepared in dimethyl sulfoxide and stored at 4°C until use. G-Glucose (analytical, reagent grade) was purchased from J. T. Baker. L-Glucose, trypsin (type III), soy bean trypsin inhibitor, dimethyl maleic anhydride, pCMB, and Triton X-100 were purchased from Sigma Chemical Co.

Dihydrocytochalasin B used as competitor in the cytochalasin B binding assays was prepared by a modified method of Aldridge et al. (1976). The abbreviation used is: pCMB, p-chloromercuribenzoate.
Maleic Anhydride, EDTA, pCMB, and Triton X-loo-Treatments performed exactly as previously described (Lin and Spudich, 1974a, buffer (pH 8.0) containing various concentrations of pCMB. After 30 min at room temperature, the reaction was quenched by addition of 60 µl of concentrated HCl. The reaction mixture was evaporated to dryness in a stream of N2, and 30 µl of solution was added to the residue. Dihydrocytochalasin B, the predominant reaction product, was extracted from the aqueous solution with several volumes of chloroform and then purified by repeated chromatography on thin layer plates (Brinkmann SIL G-200 UV254), using chloroform:ethyl acetate (1:1) as developing solvent (see Fig. 1, Lin et al., 1974). The purified compound, when chromatographed on thin layer sheets (Eastman No. 5090 silica gel, with fluorescent indicator) using the above solvent, gave a single spot with the same Rf (0.43) as that of authentic dihydrocytochalasin B generously provided by Dr. D. C. Aldridge of the Imperial Chemical Industries. The amount of contaminating cytochalasin B in this preparation was less than 1%, determined by the lack of measurable inhibition of sugar transport in human red cells and 3T3 cells at 10-8 M dihydrocytochalasin B.  

This compound has the correct molecular weight of dihydrocytochalasin B (481) as determined by mass spectrometry using a Hitachi-Perkin-Elmer RMU6 instrument. As in the case of cytochalasin B, dihydrocytochalasin B does not show any maxima in the accessible range of its ultraviolet spectrum. The end absorption of dihydrocytochalasin B measured at 260 nm was found to be proportional to its concentration over the range of 1 to 25 µM. It therefore was possible to determine the concentration of dihydrocytochalasin B solutions spectrophotometrically, in the manner described for cytochalasin B solutions (Lin et al., 1974). Dihydrocytochalasin B stored in dimethyl sulfoxide or ethanol at 0° for at least 6 months showed no changes in its chromatographic behavior or its binding properties to red cell membranes.

Preparation of Red Cell Membranes—Human red cells were isolated from human blood supplied by the Baltimore branch of the American Red Cross. Bovine and rabbit red cells were isolated from the blood of animals supplied by the American Red Cross. Bovine and rabbit red cells were isolated from the blood of animals supplied by the American Red Cross. Bovine and rabbit red cells were isolated from the blood of animals supplied by the American Red Cross. Bovine and rabbit red cells were isolated from the blood of animals supplied by the American Red Cross.

Treatment of Red Cells and Membranes with Trypsin, Dimethyl Maleic Anhydride, EDTA, pCMB, and Triton X-100. Treatment of red cells and with various chemicals were performed essentially as previously described (Lin and Spudich, 1974a, 1974c). In the trypsin experiment, 5 x 107 bovine red cells or ghosts per ml were incubated in buffer (Buffer A with 0.15 M NaCl, at 4°, and then lysed in Buffer A as described by Dodge et al. (1963). The red cell membranes (ghosts) were then collected by centrifugation (15,000 rpm, 20 min, 4°) in a Beckman J21B rotor, and washed four to six times with Buffer A until they became pearl white in color. The ghosts were stored at 4° for up to 1 week.

Cell counts were determined with a hemacytometer. Typically, 1 ml of packed human, bovine, and rabbit red cells contains 5.2 x 1010, 1.1 x 1011, and 1.2 x 1011 cells, respectively. The number of ghosts in 1 ml of packed ghosts is approximately the same as the number of cells in 1 ml of packed red cells.

Treatment of Red Cells and Membranes with Trypsin, Dimethyl Maleic Anhydride, EDTA, pCMB, and Triton X-100. Treatment of red cells and with various chemicals were performed essentially as previously described (Lin and Spudich, 1974a, 1974c). In the trypsin experiment, 5 x 107 bovine red cells or ghosts per ml were incubated in buffer (Buffer A with 0.15 M NaCl for cells, Buffer A for ghosts) containing 0.1 mg of trypsin/ml at 37°. After 30 min, trypsin inhibitor (0.1 mg/ml) was added to the reaction mixture. The cells or ghosts were collected by centrifugation, washed once with fresh buffer, and then used in cytochalasin B binding assays.

Selective extraction of membrane proteins from human and bovine red cell membranes by dimethyl maleic anhydride or EDTA was performed exactly as previously described (Lin and Spudich, 1974a, 1974c).

In the pCMB experiment, human or bovine ghosts (5 x 107 ghosts/ml) were incubated in room temperature in 0.07 M glycylglycine buffer (pH 6.0), containing various concentrations of pCMB. After 30 min, the ghosts were collected by centrifugation, washed twice with fresh buffer containing no pCMB, and then assayed for cytochalasin B binding activity. In this experiment 0.07 M glycylglycine buffer (pH 8.0) was also used in the binding assay.

Triton extraction of ghosts was performed as described by Yu et al. (1973). One volume of human or bovine ghosts was added to 5 volumes of 56 mM sodium borate buffer (pH 8.0) containing various concentrations of Triton X-100, at 0°. After 20 min, the membrane residue was collected by centrifugation, washed once in buffer containing no Triton, and then assayed for cytochalasin B binding activity.

Cytochalasin B Binding Assay—Binding of cytochalasin B to red cells and membranes was determined by the centrifugation method previously described (Lin and Spudich, 1974a). Briefly, a 200-µl sample containing 1.3 x 108 human ghosts or 1.1 x 109 bovine ghosts was added to 500 µl of buffer (Buffer A, unless specified otherwise) containing [3H]cytochalasin B or [3H]inulin and the suspension was incubated at room temperature. After 15 min, the suspension was centrifuged at 4° for 15 min at 7,000 rpm (for intact cells or ghosts) or 15,000 rpm (for extracted membranes) in a JA-20 rotor in a Beckman J21B centrifuge. The supernatant was carefully removed from the cell or membrane pellet and the radioactivity of the two fractions was determined by scintillation counting. Concentration of free cytochalasin B was calculated from the radioactivity of the supernatant; bound cytochalasin B was calculated from the radioactivity of the pellet, after subtracting trapped counts measured in [3H]inulin controls.

High affinity cytochalasin B binding described in this paper is defined as binding measured at low cytochalasin B concentrations (<10-8 M). At these drug concentrations, the level of low affinity cytochalasin B binding, determined as described previously, is less than 30 and 1% of the total binding for human and bovine ghosts, respectively.

RESULTS

High Affinity Cytochalasin B Binding Sites in Human Ghosts—We have previously reported that addition of increasing amounts of d-glucose to the binding assay media resulted in increasing inhibition of high affinity cytochalasin B binding to human ghosts (Lin and Spudich, 1974a). These results could be interpreted as competitive binding of cytochalasin B and the sugar to the same binding site on the sugar transport protein. Furthermore, the experiment also showed that inhibition of cytochalasin B binding did not reach 100% even at high concentrations of the sugar, indicating that the human ghost has high affinity cytochalasin B binding sites which are unrelated to sugar transport. We examined this possibility in greater detail by Scatchard plot analysis of the binding of cytochalasin B in the presence and absence of d-glucose (Fig. 1). Consistent with our earlier report, binding of cytochalasin B in the absence of the sugar gave a binding curve with a slope corresponding to Keq ≈ 10-7 M; extrapolation of the curve to the abscissa gives an intercept corresponding to about 3 x 108 binding sites per ghost. In addition, the two points obtained at the lowest cytochalasin B concentrations indicate that a small

![Fig. 1. Scatchard plots of the binding of cytochalasin B (CB) to human ghosts. Ghosts were assayed in the absence (●) or presence of 0.7 M d-glucose (○), or 7 x 10-5 M dihydrocytochalasin B (▲).](http://www.jbc.org/content/168/1/5465/F1)

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* S. Lin, D. C. Lin, and M. Flanagan, manuscript in preparation.
  
* S. J. Atlas and S. Lin, manuscript in preparation.
number of binding sites with $K_D < 10^{-7}$ M may be present. In the presence of 0.07 M D-glucose, the slope of the binding curve changes to a value corresponding to $K_D \approx 10^{-8}$ M and an intercept corresponding to $5 \times 10^4$ binding sites per ghost. Since the $K_D$ of D-glucose for the sugar transport protein is in the millimolar range (Stein, 1967), the concentration of D-glucose used in this experiment should have inhibited the competitive binding of cytochalasin B to transport-related sites by close to 100%. The binding curve shown in Fig. 1 therefore must represent the binding of cytochalasin B to sites which are unrelated to sugar transport.

Dihydrocytochalasin B has no significant effect on sugar transport in the human red cell and in 3T3 cells at concentrations as high as $10^{-7}$ M. However, this compound has a small but significant inhibitory effect on cytochalasin B binding to human ghosts at concentrations as low as $10^{-7}$ M (Lin and Spudich, 1974a). These results suggest that dihydrocytochalasin B competes with cytochalasin B for certain binding sites which are unrelated to sugar transport. We examine this possibility in greater detail in the following experiment. Fig. 1 shows that when cytochalasin B binding was measured in the presence of high concentrations of dihydrocytochalasin B ($7 \times 10^{-5}$ M), there is a small but significant decrease in both the slope and the extrapolated intercept of the binding curve (Fig. 1). In addition, the binding curve is linear throughout the range of cytochalasin B concentrations used in this experiment. Hence, the binding curve obtained in the presence of dihydrocytochalasin B represents the binding of cytochalasin B to sites which are related to sugar transport.

If D-glucose and dihydrocytochalasin B inhibit cytochalasin B binding by affecting two different types of sites, then the inhibitory effect of the two compounds should be additive. Data shown in Table I show that this is indeed the case. Whereas addition of either competitor to the binding assay media resulted in partial inhibition of cytochalasin B binding, addition of both compounds resulted in almost complete inhibition. In the control experiment in which an excess of unlabeled cytochalasin B was used as competitor, between 5 and 10% of the binding occurring under these conditions was found to be of the unsaturable (low affinity) type.

**Presence of High Affinity Cytochalasin B Binding Sites in Bovine and Rabbit Ghosts** — Since bovine red cells are essentially impermeable to glucose (Laris, 1958), membranes (bovine ghosts) prepared from these cells should only have cytochalasin B binding sites which are not related to sugar transport. We found that this was indeed the case: D- and L-glucose had no inhibitory effect on high affinity cytochalasin B binding to bovine ghosts whereas dihydrocytochalasin B inhibited this type of binding by close to 100% (Fig. 2A). The Scatchard plot of the binding data for the control experiment (Fig. 2B) is similar to the plot representing binding of cytochalasin B to human ghosts measured in the presence of excess D-glucose (Fig. 1). The slope of the binding curve corresponds to a $K_D$ of $2 \times 10^{-8}$ M and the extrapolated intercept at the abscissa corresponds to about $3 \times 10^4$ binding sites per ghost.

The binding of cytochalasin B to ghosts prepared with rabbit red cells, which also lack a sugar transport system similar to that found in human red cells (Hillman et al., 1959), was measured in the presence and absence of D-glucose or dihydro-

**Table I**

*Inhibition of binding of [3H]cytochalasin B to human ghosts by D-glucose and dihydrocytochalasin B.*

| Competitor            | Inhibition of binding of [3H]cytochalasin B to ghosts |
|-----------------------|-------------------------------------------------------|
|                       | At $7 \times 10^{-8}$ M [3H]CB At $1.4 \times 10^{-7}$ M [3H]CB |%
| None                  | 0 0 |
| L-Glucose             | 0 0 |
| D-Glucose             | 53 55 |
| H$_2$CB               | 40 33 |
| L-Glucose + H$_2$CB   | 47 37 |
| D-Glucose + H$_2$CB   | 92 83 |
| Unlabeled CB          | 95 90 |

**Fig. 2.** Binding of cytochalasin B (CB) to bovine ghosts. A, bovine ghosts were assayed in the absence ( ) or presence of 0.35 M D-glucose (O), 0.35 M L-glucose (A), or $7 \times 10^{-8}$ M dihydrocytochalasin B (O). B, Scatchard plot of data presented in A.
cytochalasin B. As in the case with bovine ghosts, this membrane preparation has only cytochalasin B binding sites which bind dihydrocytochalasin B but not α-glucose (Fig. 3).

Properties of High Affinity Cytochalasin B Binding Sites in Bovine Ghosts—The substrate specificity of cytochalasin B binding sites in bovine ghosts is presented in Table II. All cytochalasins tested inhibited cytochalasin B binding by nearly 100%, showing that the binding sites can use all of them as substrates in the binding reaction. Consistent with data presented in Fig. 2, D and L-glucose have no inhibitory effect on cytochalasin B binding to these sites.

For comparison, the effects of different competitors on cytochalasin B binding to human ghosts are also presented in Table II. All of the cytochalasins affected cytochalasin B binding, but inhibition did not reach 100% except in the case of cytochalasin B. These results show that human red cell membranes have some binding sites which can use different cytochalasins as binding substrates. α-Glucose inhibited cytochalasin B binding by only 18% in this experiment, indicating that at this cytochalasin B concentration (7 × 10⁻⁹ M), only a small fraction of the cytochalasin B was bound to sugar transport-related sites.

The rate of binding of cytochalasin B to bovine ghosts was measured at 4°, 23°, and 37° (Fig. 4). At all three temperatures, binding reached maximal levels by the time the first samples were taken. At 4° and 23°, incubation for up to 2 h did not significantly affect the amount of bound cytochalasin B. At 37°, however, there was a sharp decrease in the amount of bound cytochalasin B with increasing incubation time. After 2 h of incubation at 37°, binding was down to 10% of the maximal level. This decrease in binding could be attributed to inactivation of cytochalasin B binding sites. Control experi-

![Fig. 3. Binding of cytochalasin B (CB) to rabbit ghosts. Samples containing 1.2 × 10⁶ ghosts were assayed in the absence (○) or presence of 0.35 M α-glucose (○), 0.35 M L-glucose (△), or 7 × 10⁻⁴ M dihydrocytochalasin B (□).](image)

![Fig. 4. Rates of binding and dissociation of cytochalasin B (CB) from bovine ghosts. A, 200-μl samples containing 1.1 × 10⁶ ghosts were incubated in buffer containing 6 × 10⁻⁴ M cytochalasin B at zero time. The reaction mixtures were incubated with occasional stirring at 4° (○), 23° (●), or 37° (△). At the times indicated, ghosts were separated from the assay media by centrifugation and the amount of bound cytochalasin B was determined. B, 200-μl samples containing 1.1 × 10⁶ ghosts were incubated in 500 μl of buffer containing 6 × 10⁻⁴ M cytochalasin B, at 23°. After 20 min, the ghosts were separated from the cytochalasin B containing buffer by centrifugation and then resuspended in 500 μl of fresh buffer containing no cytochalasin B. The suspensions were incubated at 4° (○), 23° (●), or 37° (△), with occasional stirring. At the times indicated, the ghosts were separated from the buffer by centrifugation and the amount of bound cytochalasin B was determined.](image)
Cytochalasin B Binding Sites Unrelated to Sugar Transport

Fig. 5. Effect of pH and ionic strength on binding of cytochalasin B (CB) to bovine ghosts. A, ghosts were incubated in 5 mM sodium phosphate buffer containing $6 \times 10^{-8}$ M cytochalasin B at the pH indicated (pH was adjusted by addition of NaOH or HCl). B, ghosts were incubated in Buffer A containing $6 \times 10^{-8}$ M cytochalasin B and the indicated concentration of KCl.

ments showed that ghosts which had been preincubated in buffer for 2 h at 37°C lost almost all of their capacity to bind cytochalasin B when tested in a standard assay, whereas ghosts preincubated at 4°C or 23°C for 2 h were not significantly affected.

The rate of dissociation of cytochalasin B bound to bovine ghosts was also examined (Fig. 4B). At all three temperatures tested, there was a rapid release of bound cytochalasin B when the ghosts were resuspended in buffer containing no cytochalasin B. At 4°C and 23°C, new equilibrium conditions were reached in less than 1 min. At 37°C, the amount of bound cytochalasin B continued to drop with time, presumably because of inactivation of binding sites.

The binding of cytochalasin B to bovine ghosts was determined at pH 5.0 to 9.0 (Fig. 5A). Although no sharp pH maxima were found, it is apparent that the binding reaction was enhanced by acidic conditions. The binding of cytochalasin B to the membranes was also examined in buffers containing 0 to 1 M KCl (Fig. 5B). It was found that a small but appreciable increase in binding occurred when the ionic strength of the buffer was increased.

The amount of cytochalasin B bound to intact bovine red cells was about the same as that bound to an equivalent number of bovine ghosts (Fig. 6A), indicating that all of the high affinity cytochalasin B binding sites of these cells are associated with the cell membrane. When the intact cells were treated with trypsin, there was no decrease in their capacity to bind cytochalasin B (Fig. 6A). In contrast, when membranes were treated with trypsin under conditions where both sides of the membranes were accessible to the proteolytic enzyme, binding capacity decreased by about 70% (Fig. 6B). These results suggest that the high affinity cytochalasin B binding sites are located in membrane associated proteins which are exposed primarily, if not exclusively, to the cytoplasmic side of the cell membrane.

The binding of cytochalasin B to sugar transport-related sites in human ghosts are inhibited by low concentrations of the sulfhydryl reagent pCMB (Lin and Spudich, 1974a). In contrast, binding of cytochalasin B to bovine ghosts was not

Fig. 6. Effect of trypsin on cytochalasin B (CB) binding sites in bovine red cells and ghosts. A, samples contained untreated ghosts (△) or red cells which had been incubated in the absence (●) or presence (○) of 0.1 mg/ml of trypsin at 37°C for 30 min. B, samples contained ghosts which had been incubated in the absence (●) or presence (○) of 0.1 mg/ml of trypsin at 37°C for 30 min.
affected by this reagent except at a very high concentration (10^{-2} M) (Fig. 7). At 10^{-6} M pCMB, binding of cytochalasin B to human ghosts was inhibited by about 70% whereas binding of cytochalasin B to bovine ghosts was not affected at all.

We have previously found that human red cell membranes extracted with dimethyl maleic anhydride still contain all of the sugar transport-related high affinity cytochalasin B binding sites, as measured with an equilibrium dialysis assay (Lin and Spudich, 1974c). When we treated bovine ghosts with dimethyl maleic anhydride, we found that the membrane residua no longer bound cytochalasin B with high affinity (Fig. 8). Similarly, extraction with EDTA, which also has no effect on sugar transport-related sites in the human red cell (Lin and Spudich, 1974a), led to a complete loss of cytochalasin B binding activity. Whether dimethyl maleic anhydride and EDTA released or inactivated the cytochalasin B binding sites in bovine ghosts is not known; only a small fraction of the total cytochalasin B binding activity can be recovered in the extracted protein fraction (determined with an equilibrium dialysis cytochalasin B binding assay).

Since cytochalasin B binding sites which are unrelated to sugar transport are sensitive to EDTA treatment, human red cell membranes extracted with this reagent should contain only sugar transport-related cytochalasin B binding sites. Data presented in Fig. 9 show that this was indeed the case. Whereas d-glucose inhibition of cytochalasin B binding to untreated human ghosts was about 70%, inhibition of cytochalasin B binding to EDTA-extracted membranes reached 90%.

Consistent with this result is our earlier observation that cytochalasin B binding to dimethyl maleic anhydride-ex-
concentrations. In fact, this conclusion is based on the finding that sugars do not be found in bovine and rabbit red cells, which are known to B binding sites (henceforth referred to as Class II sites) in human, bovine, and rabbit red cells. These sites are distinct from the sugar transport-related cytochalasin B binding sites (Class I sites) described in earlier reports (Lin and Spudich, 1974a, 1974c) for the following reasons. First, Class II sites can from the sugar transport-related cytochalasin B binding sites of bovine ghosts. Samples contained ghosts extracted with the indicated concentration of Triton.

Yu et al. (1973) have shown that extraction of human red cell membranes with Triton X-100 has the opposite effect as extraction with dimethyl maleic anhydride; membrane proteins extracted by dimethyl maleic anhydride are not extracted by this detergent. We tested the effect of Triton extraction on bovine red cell membranes and found that increasing Triton concentration in the extraction buffer resulted in increasing loss of cytochalasin B binding activity (Fig. 10). Similar results were also obtained when human ghosts were extracted with this detergent. Thus, cytochalasin B binding sites in both types of red cells are either released or inactivated by the Triton treatment.

**DISCUSSION**

We have identified a new class of high affinity cytochalasin B binding sites (henceforth referred to as Class II sites) in human, bovine, and rabbit red cells. These sites are distinct from the sugar transport-related cytochalasin B binding sites (Class I sites) described in earlier reports (Lin and Spudich, 1974a, 1974c) for the following reasons. First, Class II sites can be found in bovine and rabbit red cells, which are known to lack a sugar transport system similar to that found in human red cells (Laris, 1958; Hillman et al., 1959). Second, unlike Class I sites, Class II sites apparently do not bind p-glucose. This conclusion is based on the finding that sugars do not inhibit binding of cytochalasin B to these sites even at high concentrations. In fact, p-glucose seems to have a small but significant enhancing effect on cytochalasin B binding to these sites. This effect, however, is not stereospecific because L-glucose also produces a similar effect. Third, binding of cytochalasin B to Class II sites can be completely inhibited by the presence of an excess of dihydrocytochalasin B in the binding assay media, indicating that the latter compound is also a substrate for these sites. Dihydrocytochalasin B has been found to be ineffective in inhibiting sugar transport into human red cells and 3T3 cells. Furthermore, this compound, even at high concentrations, does not reduce the inhibitory effect of cytochalasin B on sugar transport, showing that it does not bind to the Class I sites of these cells.

Our studies on cytochalasin B binding to bovine ghosts revealed that there are other differences between Class I and Class II sites besides substrate specificity. The $K_D$ for Class II sites is significantly lower than that of Class I sites, indicating that cytochalasin B has a higher affinity for the former. Inhibition studies using pCMBS showed that free sulfhydryl groups are essential for binding of cytochalasin B to Class I sites but not to Class II sites. Finally, Class I sites are not removed from the red cell membrane by dimethyl maleic anhydride or EDTA extraction whereas Class II sites are either removed or inactivated by these treatments.

We have previously reported that human red cell membranes extracted with EDTA or dimethyl maleic anhydride retained all of the high affinity cytochalasin B binding activity of ghosts (Lin and Spudich, 1974a, 1974c). We report here that human red cells have Class II sites which are either removed or inactivated by the extraction procedures. This apparent inconsistency can be explained by the lability of the Class II sites. Since comparison of cytochalasin B binding capacity of human red cell membranes before and after extraction was measured by equilibrium dialysis at room temperature for 22 h (Lin and Spudich, 1974a, 1974c), it is possible that all of the Class II sites in the untreated ghost was inactivated under those conditions and that binding of cytochalasin B to only Class I sites was actually measured. This possibility is supported by the finding that bovine ghosts did not bind any cytochalasin B with high affinity when assayed under similar conditions.

The molecular identity of the Class II sites in red cells remains to be determined. However, certain speculations on the biological function of these sites may be made from data on the biological effects of cytochalasin B and dihydrocytochalasin B on other cell types. We have found that although dihydrocytochalasin B does not inhibit sugar transport in human red cells and 3T3 cells, it has similar effects as cytochalasin B in altering the morphology of 3T3 and glial cells, and in inhibiting platelet-mediated blood clot retraction, photodinesis and cytoplasmic streaming in plant cells, nerve growth cone activity, and cytokinesis and membrane ruffling in 3T3 cells. Thus, the observation that Class II sites apparently bind both cytochalasin B and dihydrocytochalasin B suggests that the sites are directly or indirectly related to cell motility and morphology, and not to sugar transport. Indeed, cytochalasin B has been shown to increase the osmotic fragility and mechanical deformability of human red cells (Beck et al., 1972), and to decrease the rigidity of the red cell membrane, as judged by electron paramagnetic resonance studies (Huestis and McConnell, 1974). It is possible that these effects are caused by the binding of the drug to Class II sites, although more detailed studies on the dependence of these effects on drug concentrations must be performed before a definite correlation can be made.

High affinity sites which apparently bind cytochalasin B and dihydrocytochalasin B, but not p-glucose, are not unique to red cells. We have previously found that high affinity binding of [3H]cytochalasin B to normal and transformed 3T3 cells is not affected by sugars (Atlas and Lin, 1976), but is inhibited by an excess of unlabeled cytochalasin B or dihydrocytochalasin B. The lack of effect of p-glucose on high affinity binding of cytochalasin B to fat cells (Czech, 1976) and Novi-

* S. Lin, unpublished results.
Cytochalasin B Binding Sites Unrelated to Sugar Transport

Cytochalasin B binding sites unrelated to sugar transport have also been reported. Although the effect of dihydrocytochalasin B on cytochalasin B binding was not determined in these studies, high affinity binding sites for cytochalasin D have been found in platelets (Puszkin et al., 1973) and in several lines of cultured animal cells (Tannenbaum et al., 1975). The relationship between these sites and the Class II sites in red cells remains to be determined.

The number of Class II sites in red cells (10^4 to 10^5/cell) is substantially lower than that found in 3T3 cells (10^6 to 10^7/cell) even when difference in cell size is taken into account. If these sites are indeed related to cell motility and morphology, it is perhaps not surprising to find that red cells, which are essentially nonmotile, have a relatively small number of these sites.

There is a considerable amount of evidence from in vivo and in vitro experiments indicating that cytochalasin B affects cell motility and morphology by interacting with actomyosin-like proteins in the cell (Wessells et al., 1971; Spudich and Lin, 1972; Spudich, 1972; Hartwig and Stossel, 1976; Weihing, 1976). In the red cell, a network of proteins associated with the cytoplasmic side of the cell membrane is thought to play an important role in defining and controlling the shape of the cell (Steck, 1974). These proteins can be eluted from the membrane with EDTA and have been shown by isoelectric focusing followed by sodium dodecyl sulfate gel electrophoresis to contain several distinct forms of spectrin, actin, and several minor polypeptides (Bhakdi et al., 1974). If the loss of cytochalasin B binding activity caused by EDTA extraction of red cell membranes is not due to inactivation of Class II sites, then it is likely that one or more of these eluted proteins contain high affinity cytochalasin B binding sites.

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