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REVERSIBLE ASSOCIATION OF CYTOCHALASIN B WITH THE HUMAN ERYTHROCYTE MEMBRANE

INHIBITION OF GLUCOSE TRANSPORT AND THE STOICHIOMETRY OF CYTOCHALASIN BINDING

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SUMMARY

The effect of cytochalasin B upon glucose translocation across the human erythrocyte membrane has been investigated both by the classical Ørskov method and by the O_2 uptake method recently described (Taverna, R. D. and Langdon, R. G. (1973) *Biochim. Biophys. Acta* 298, 412–421 and 422–428) .Cytochalasin B was found to be a powerful noncompetitive inhibitor of glucose transport which had a kinetically determined K_i of 0.70 μM when measured by the Ørskov method and 0.34 μM when measured by the O_2 uptake method. Equilibrium binding studies revealed that there are approx. 330 000 cytochalasin binding sites per erythrocyte, and that the K_{Diss} of cytochalasin bound to these sites is 49 nM. It is suggested that one of the major electrophoretically identifiable erythrocyte membrane proteins may be the cytochalasin binding site.

INTRODUCTION

Since Carter's initial report[1] that the mold metabolite cytochalasin B reversibly inhibited cytoplasmic cleavage of dividing mouse fibroblasts, numerous diverse observations concerning the activity and mode of action of this compound have been reported.

Schroeder[2] observed that inhibition of HeLa cell mitosis by cytochalasin B was accompanied by disruption of microfilaments. Since then, cytochalasin B has been reported to inhibit reversibly a variety of types of cell movements including sorting of embryonic cells[3], phagocytosis by human leukocytes[4], pinocytosis in liver cells[5], cell motility[6], contraction of embryonic heart cells[7], and to produce ultrastructural alterations interpreted as disruption of the microfilaments.

Although the relationship of the F-actin-like microfilaments to cellular activity is not entirely clear, it is apparent that cellular movements require an energy

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source and that cytochalasin B might inhibit mobility by interference with energy metabolism. Wagner *et al.*[5] demonstrated that iodoacetate poisoning of liver cells resulted both in inhibition of respiration and of pinocytosis in liver cells; in contrast, cytochalasin B inhibited pinocytosis without affecting respiration. On the other hand, Sanger and Holtzer[8] found that inhibition of cell aggregation by cytochalasin B was accompanied by decreased conversion of glucosamine to mucopolysaccharides, and they suggested that cytochalasin B blocked the formation of a surface component which turns over rapidly. Furthermore, it has been reported very recently that cytochalasin B powerfully inhibits monosaccharide translocation into several cultured cells[9] without affecting respiration or the active accumulation of amino acids.

The relationship between microfilament disruption and inhibition of monosaccharide translocation has remained somewhat obscure. Very recently, while this work was in progress, Kletzein and Perdue[10] presented convincing evidence that cytochalasin B inhibited the uptake of labeled monosaccharides by cultured chick fibroblast cells at inhibitor concentrations 300-fold less than those required to disrupt microfilaments; their data suggested that cytochalasin B acted as a competitive inhibitor of monosaccharide transport across the plasma membranes of the fibroblasts.

Those cells previously examined had been capable of pinocytosis, cytokinesis, and of metabolic turnover of their constituents. It appeared desirable to assess the effect of this agent upon a cell which is incapable of metabolic turnover, or of cellular movement but which retains facilitated diffusion of monosaccharides; the mature human erythrocyte is almost ideally suited for this purpose because it is anucleate, does not undergo any recognized energy-requiring cellular movements, but does possess a facilitated diffusion system for monosaccharides which resembles those in liver[9], fibroblasts[11], heart cells[12], and skeletal muscle[13]. Moreover, although the cell is not motile and does not display active pinocytosis, it does have microfilaments[14] associated with its membrane, and can be induced to undergo a non-energy-requiring endocytotic process[15].

By employing a method previously described for measuring the kinetic parameters of glucose translocation in human erythrocytes[16], we have demonstrated that cytochalasin B is a noncompetitive inhibitor of this process, having a kinetically determined K_i of 0.34 μM. Equilibrium binding studies revealed that there are approx. 330 000 cytochalasin B binding sites per erythrocyte; the K_{Diss} for cytochalasin B bound to these sites was approx. 49 nM. Cytochalasin B was found to have no effect on endocytosis in this cell when employed at concentrations sufficient to completely inhibit transport.

MATERIALS AND METHODS

Glucose oxidase purchased from Sigma Chemical Co. was purified as previously described[16,17]. Crystalline catalase was obtained from Worthington Biochemical Corporation. Anhydrous D-glucose was a product of Pfanstiehl Laboratories. DEAE-cellulose was purchased from Schleicher and Schull Company. Cytochalasin B was obtained from Imperial Chemical Industries, Ltd. Silica gel thin-layer plates, F-254, were purchased from Brinkman Instruments, Inc.

Glucose translocation through human erythrocyte membranes was measured by two methods, a light scattering method originally described by Ørskov[18], and an enzymatic method employing an oxygen electrode which we have previously described in detail[16]. Human erythrocytes were obtained from whole blood freshly drawn from normal donors; ethylenediaminetetracetate, 1 mg/1 ml, was used as an anticoagulant. Aliquots of 2 ml of whole blood were added to 10 ml of isotonic NaCl solution at room temperature and the suspended erythrocytes were sedimented by centrifugation at $300 \times g$ for 10 min. The supernatant solution, which contained most of the leukocytes, was aspirated from the loosely packed erythrocytes. The erythrocytes were twice resuspended in 10 ml of fresh isotonic saline and recovered by centrifugation at $600 \times g$ for 10 min. The washed erythrocytes were chilled to 0 °C, resuspended in isotonic Krebs–Ringer phosphate buffer, and again harvested by centrifugation at $600 \times g$ for 5 min. The collected cells were retained at 4 °C until further use.

Pink erythrocyte ghosts containing glucose oxidase and catalase in their interior spaces were prepared as previously described. As we have shown earlier, the kinetics of glucose influx into these enzyme-containing ghosts may be conveniently measured with an oxygen electrode; the rate of O_2 uptake is directly related to the rate of glucose entry into the glucose oxidase-containing space. The rate of glucose permeation was measured at 15 °C utilizing ambient glucose concentrations ranging from 1.5 to 76 mM.

The effect of cytochalasin B on glucose entry was examined by mixing 5-μl aliquots of cytochalasin B solutions in ethanol or dimethylsulfoxide with 1 ml portions of glucose oxidase- and catalase-containing ghosts which had been warmed at 20 °C. Aliquots of 0.65 ml were introduced into the oxygen electrode chamber and allowed to attain thermal equilibrium (15 °C) prior to injection of 25 μl of D-glucose solution; O_2 uptake was then recorded continuously on a strip-chart recorder. In early experiments more rapid rates of O_2 consumption were observed when ethanolic solutions of cytochalasin B were utilized than when the compound was dissolved in dimethylsulfoxide; it was ascertained that this resulted from peroxidation of ethanol by catalase in the presence of H_2O_2 generated by glucose oxidase; although it was possible to correct for this artifact, it was found more convenient to utilize dimethylsulfoxide solutions of cytochalasin B, and this was done routinely in the experiments reported here unless otherwise noted. Dimethylsulfoxide itself at the concentrations employed here was found to have no effect on transport. Appropriate control experiments revealed that cytochalasin B, in the concentrations employed here, had no effect upon glucose oxidase.

For measuring the effect of cytochalasin B upon glucose translocation by the Ørskov light scattering method as modified by Sen and Widdas[19], 1-ml aliquots of washed erythrocyte suspensions were mixed with 10 ml of Krebs–Ringer phosphate buffer containing 75 mM glucose. After incubation at 37 °C for 10 min, the cells were collected by centrifugation at $1000 \times g$ for 10 min at room temperature. The pellets were resuspended in 75 mM glucose in Krebs–Ringer phosphate buffer and maintained at 22 °C until used in transport experiments. Into the cuvette of the Ørskov apparatus were placed 3-ml aliquots of Krebs–Ringer phosphate buffer containing from 0–20 mM D-glucose. Into these were introduced, with a Hamilton syringe, 3-μl portions of ethanol containing 0, 0.21 mM and 2.1 mM cytochalasin B.

Into the cuvette were then injected 25-μl portions of the erythrocyte suspension. The change in light transmission with time, which is directly proportional to the change in red cell volume, was recorded continuously on a strip-chart recorder. From the tracings, the exit times at each ambient glucose and cytochalasin concentration were obtained as described by Sen and Widdas[19], and were plotted against ambient glucose concentrations as described by these authors.

The equilibrium binding of cytochalasin B to erythrocytes was investigated in the following manner. Washed erythrocytes resuspended in Krebs–Ringer phosphate buffer were warmed to 22 °C, and 1-ml aliquots of the suspension were added to 2.0-ml portions of Krebs–Ringer phosphate buffer containing various concentrations of cytochalasin B. After incubation for 5 min, the suspensions were either maintained at 22 °C or chilled to 4 °C and centrifuged at $10000 \times g$ for 5 min. The supernatant solutions were withdrawn and mixed with glucose oxidase- and catalase-containing ghosts. These mixtures were then placed in the oxygen electrode chamber, D-glucose was added to each, and the rates of O_2 uptake were recorded. The concentration of cytochalasin B in each supernatant fraction could then be calculated by comparison with the rates of O_2 uptake of the same preparations of glucose oxidase- and catalase-containing ghosts in the presence of measured known concentrations of cytochalasin B. In each experiment, the number of washed erythrocytes per ml of original suspension was carefully measured by counting four replicate samples of each suspension in a Coulter counter. This allowed precise calculation of the number of cytochalasin binding sites per erythrocyte. 20 μl cytochalasin B (1 mg/ml) in dimethylsulfoxide or ethanol was placed on 20 cm × 9 cm F-254 silica gel thin-layer plates and chromatographed in a chloroform–methanol–water solvent system (65:25:4, v/v/v). The plates were allowed to air dry. A single spot was observed when visualized under 253.7-nm wavelength light source. The resulting R_F was 0.759.

RESULTS

In Fig. 1 is illustrated the effect of cytochalasin B upon glucose translocation through the erythrocyte membrane as measured by the Ørskov[18] exit method of Sen and Widdas[19]. It is evident that at each ambient glucose concentration, the exit time is prolonged in the presence of 2.1 μM cytochalasin B. The value of the intercept of each line with the abcissa is 3 mM; this intercept corresponds to the half-saturation constant (K_T) of the transport process for glucose. It is apparent that it is not altered by cytochalasin B. The intercept on the ordinate corresponds to the exit time under zero *trans* conditions, and is proportional to the reciprocal of the maximal velocity of glucose efflux; it is evident that cytochalasin B added to the external surface of the erythrocyte inhibited efflux of glucose from the *trans*-side, both in the presence and absence of glucose on the *cis*-side of the membrane. The fact that the maximal velocity of glucose transport diminished in the presence of cytochalasin B without alteration of K_T suggests that cytochalasin B is a non-competitive inhibitor of glucose transport. From these experiments, a tentative value for the half-saturation constant (K_i) for cytochalasin B can be calculated; it is 0.65 μM.

To obtain a more precise value for the cytochalasin K_i, the exit times from

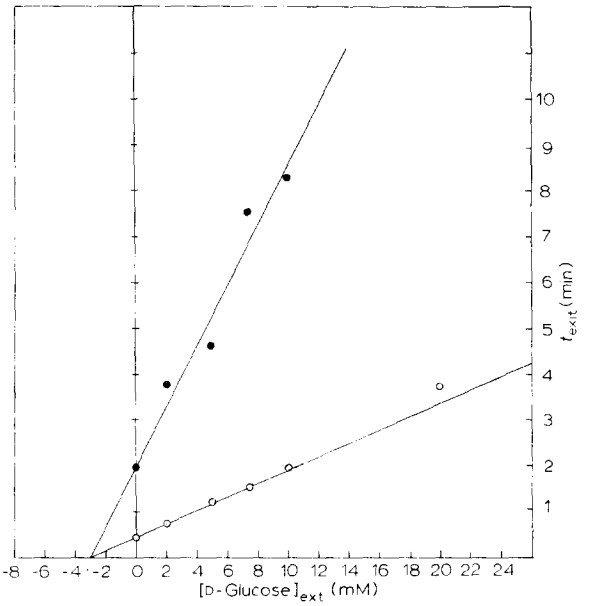

Fig. 1. Effect of cytochalasin B upon glucose translocation through the erythrocyte membrane as measured by the Ørskov method. Erythrocytes containing 75 mM D-glucose were injected into the cuvette which contained increasing concentrations of D-glucose alone (○—○) or D-glucose *plus* 2.1 μM cytochalasin B (●—●). All experiments were done at 22 °C. Each solution contained 0.15% ethanol.

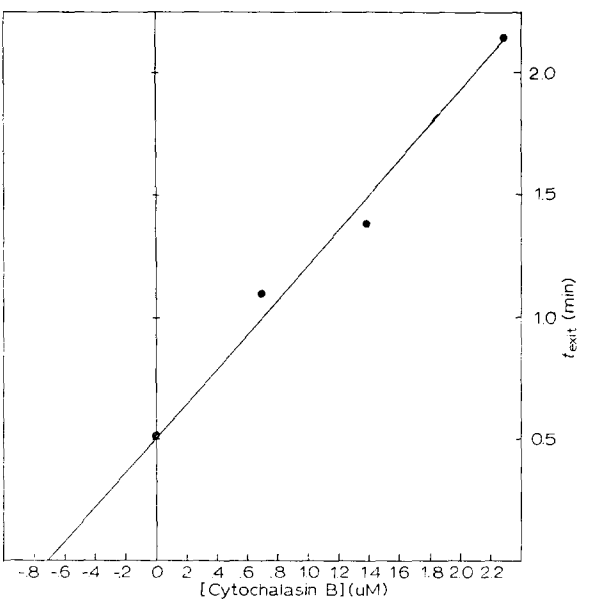

Fig. 2. Effect of varying concentrations of cytochalasin B upon glucose translocation through the human erythrocyte membrane as measured by the Ørskov method. Erythrocytes containing 75 mM D-glucose were injected into the cuvette which contained increasing concentrations of cytochalasin B dissolved in glucose-free suspending medium. All experiments were done at 22 °C.

glucose-loaded erythrocytes were measured under zero *trans* conditions as a function of cytochalasin B concentrations. The results are depicted in Fig. 2. Under these circumstances the value of the intercept with the abcissa is K_i; its value is 0.70 μM.

Since experiments employing the Ørskov exit method are restricted to adding glucose and cytochalasin B on opposite sides of the membrane, and because the use of glucose oxidase- and catalase-containing ghost allowed more flexibility

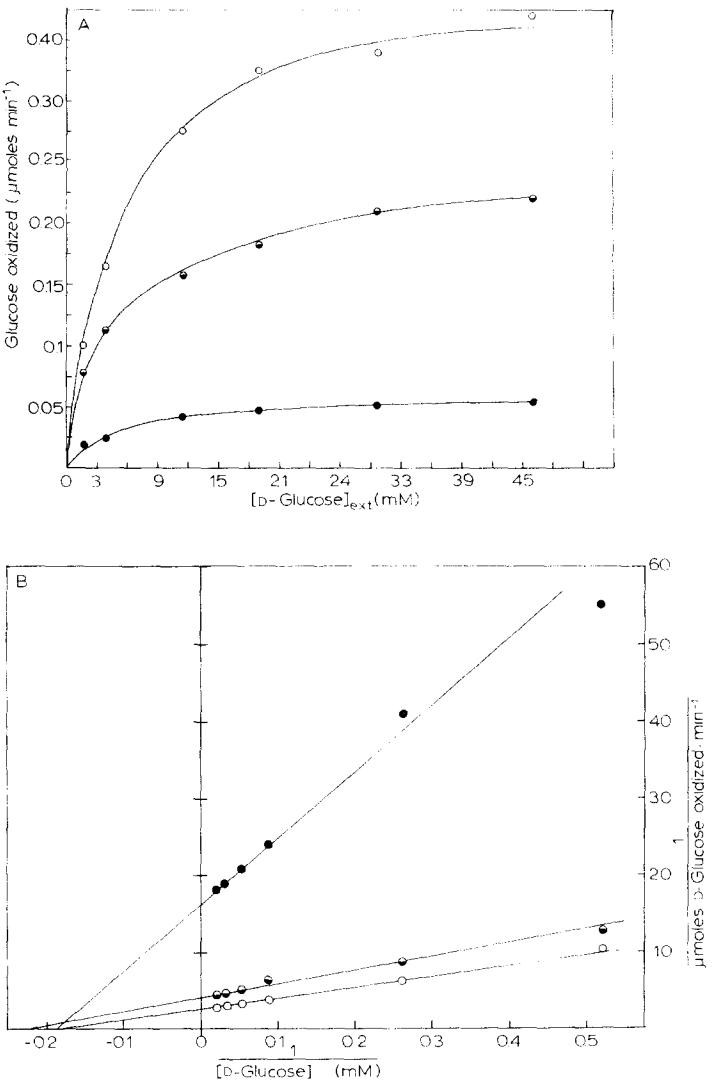

Fig. 3. Effect of cytochalasin B upon glucose translocation through the membranes of glucose oxidase- and catalase-containing ghosts as a function of glucose concentration. (A) The rates of the control (○—○) are compared with those in the presence of 0.21 μM (◒—◒) and 2.1 μM (●—●) cytochalasin B. (B) The data from (A) are presented in double reciprocal form to illustrate the noncompetitive nature of the inhibition of transport by cytochalasin B. (○—○), control; (◒—◒), 0.21 μM cytochalasin B; (●—●), 2.1 μM cytochalasin B.

and precision of measurement, further experiments were conducted with the latter method.

Glucose oxidase- and catalase-containing ghost suspensions containing 0.21 μM and 2.1 μM total cytochalasin B, prepared as described in Materials and Methods, were introduced into the O_2 electrode chamber, and 25-μl aliquots of D-glucose solutions of varying concentration were injected into the stirred suspensions. The rates of O_2 uptake under steady state conditions[16] were recorded. The results, depicted in Fig. 3 show clearly that cytochalasin B is a potent inhibitor of transport, and that the degree of inhibition is related to the cytochalasin B concentration. It is also apparent that cytochalasin B appears to be a noncompetitive inhibitor of glucose influx when both glucose and cytochalasin B are added to the same side of the membrane. Nonlinear regression analysis of the data by the Wilkinson method[20] yielded a value for K_i of 0.34 μM.

Since inhibition of glucose transport by cytochalasin B was found to be noncompetitive, it was convenient to examine, at a fixed concentration of glucose, the inhibitory action of cytochalasin B over a wide range of inhibitor concentrations. In the experiments illustrated in Fig. 4, the rates of glucose transport in the presence of 35 nM to 5.2 μM cytochalasin B were examined. The sigmoidal shape of the semilogarithmic plot suggests that inhibition by cytochalasin B cannot be attributed to a simple partitioning of cytochalasin B between membrane and solution, but must represent equilibrium binding to a finite number of sites. A Hill-type plot of the data in Fig. 4 is presented in Fig. 5; the slope is 1.27, which suggests that the binding of cytochalasin B is noncooperative.

By utilizing the data in Fig. 4, it was possible to carry out an equilibrium binding study of cytochalasin B to erythrocytes, to determine the number of binding sites per erythrocyte, and the actual $K_{D_{ISS}}$ of the complex. As described in detail in the Materials and Methods section, measured numbers of erythrocytes were

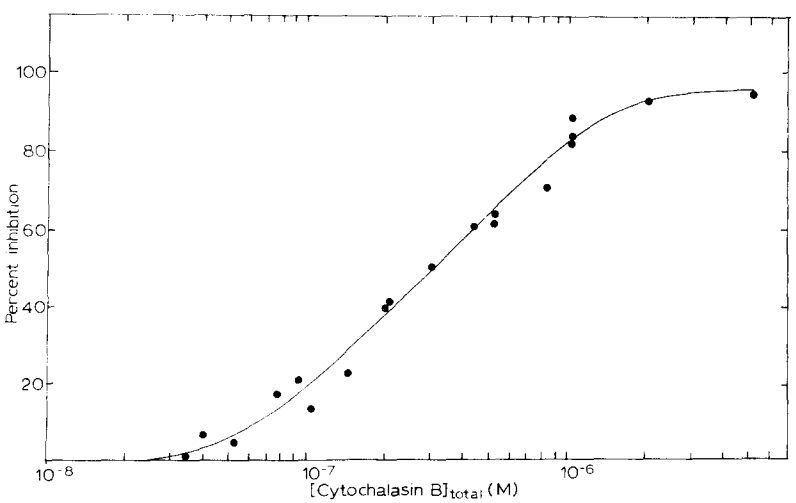

Fig. 4. Dose–response curve for inhibition by cytochalasin B of glucose translocation through glucose oxidase- and catalase-containing ghost membranes. Ambient glucose concentration was 30 mM in each experiment. The temperature was 15 °C.

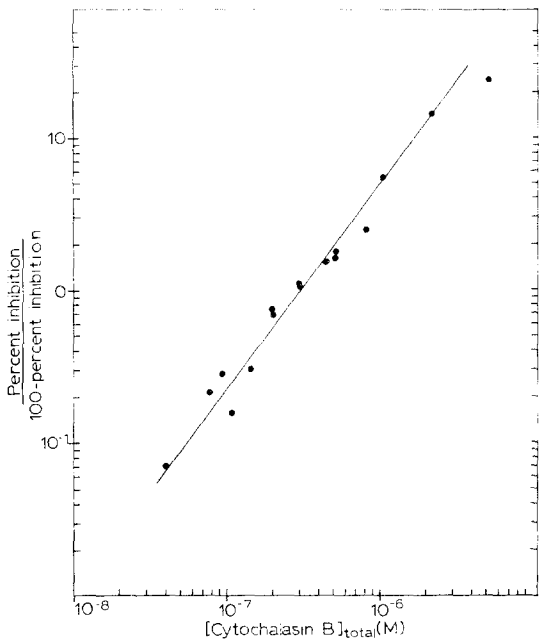

Fig. 5. Hill plot of inhibition by cytochalasin B of glucose translocation. The data from Fig. 4 were utilized to construct this figure.

incubated in cytochalasin solutions of known total concentration; following incubation, the erythrocytes were removed by centrifugation and glucose oxidase- and catalase-containing ghosts were suspended in the supernatant fluids. From the extent of inhibition of glucose transport, the free cytochalasin concentration in each original erythrocyte supernatant could be ascertained by reference to the curve of Fig. 4. By difference, the remainder of the total cytochalasin was assumed to be bound to the sedimented erythrocytes. The values for bound and free cytochalasin B were then plotted in a Scatchard plot as illustrated in Figs 6 A and B. From this, tentative values for K_{Diss} and total number of binding sites were readily derived. The latter are tabulated in Table I.

All of the above data suggested that cytochalasin was bound reversibly by the erythrocyte membrane, and that this binding was responsible for inhibition of glucose transport. If the binding were indeed reversible, it should be possible to remove the cytochalasin B and restore transport. Glucose oxidase- and catalase-containing ghosts whose glucose transport had been inhibited by exposure to 2.1 μM cytochalasin B were sedimented by centrifugation, resuspended in fresh cytochalasin-free Krebs–Ringer phosphate buffer, incubated 70 min at 20 °C, and their glucose transport activity examined. The results in Fig. 7 show that 70% of the transport activity had been restored after the first wash. The remaining points in this figure represent the results of serial washing and incubation of the originally inhibited cells. It is apparent that after four washes, essentially no inhibition was observed.

Since the dissociation of cytochalasin bound to the membrane seemed to

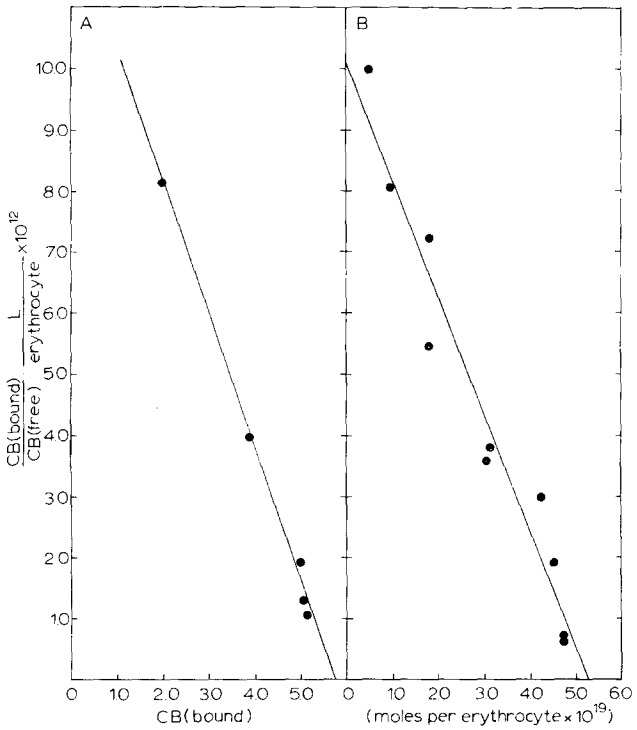

Fig. 6. Equilibrium binding of cytochalasin B (CB) to human erythrocytes at 4 °C (A) and 22 °C (B).

TABLE I

EQUILIBRIUM BINDING OF CYTOCHALASIN B TO HUMAN ERYTHROCYTES

The values for K_{Diss} and the number of cytochalasin B binding sites were obtained from Fig 6.

Temperature (°C)	K_{Diss} (*nM*)	*Cytochalasin B binding sites* (*No. per erythrocyte*)
4	45.8	$3.47 \cdot 10^5$
22	52.0	$3.18 \cdot 10^5$

be a fairly rapid process, it was desirable to obtain some estimate of the rapidity of inhibition. Glucose oxidase- and catalase-containing ghosts were introduced into the O_2 electrode chamber, O_2 uptake was initiated by addition of glucose, and after several seconds 0.65 μl of 0.15 mM cytochalasin B was injected. Its apparent inhibition was unmeasurably rapid. Numerous experiments of this type have yielded essentially the same results. In order to determine whether prolonged exposure to cytochalasin B resulted in more extensive inhibition, glucose oxidase- and catalase-containing ghosts were incubated at 20 °C in the presence of cytochalasin B for various time periods prior to measurement of glucose transport by the oxygen

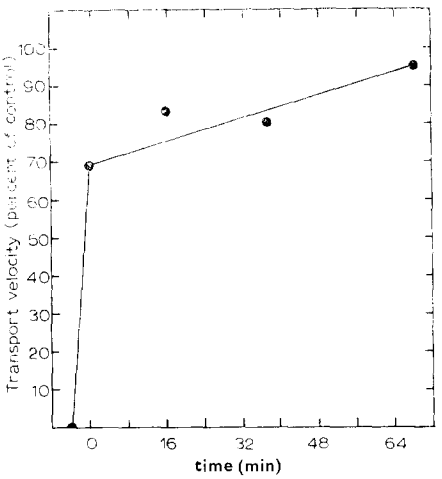

Fig. 7. Reversibility by washing of cytochalasin B inhibition of glucose transport. The recovery of glucose transport activity by glucose oxidase- and catalase-containing ghosts which initially had been treated with 2.1 μM cytochalasin B is shown as a function of incubation time at 22 °C in cytochalasin B-free Krebs–Ringer phosphate buffer.

TABLE II

INHIBITION OF GLUCOSE TRANSLOCATION BY CYTOCHALASIN B AS FUNCTIONS OF TIME AND CYTOCHALASIN B CONCENTRATION.

Cytochalasin B concentration (μM)	*Time of preincubation* (*min*)	*Transport inhibition* (%)
0.021	2.5	12
	27.5	5
0.210	0	38
	2.5	32
	23.0	38
2.100	3.0	88
	18.0	88

electrode method. The results, presented in Table II, show that the extent of inhibition is not dependent upon the time of exposure of the cells to cytochalasin B.

In order to assess the effect of cytochalasin B upon the non-energy requiring endocytosis which erythrocytes may be made to undergo, electron micrographs were obtained of hemoglobin-free erythrocyte ghosts which had been exposed to cytochalasin B and were compared with untreated ghosts. These did not reveal any effect of cytochalasin B upon the morphology of these membranes. The ghosts were then induced to undergo endocytosis by the method of Steck *et al.*[15] in the absence and presence of 2.1 and 21.0 μM cytochalasin B. Electron micrographs of thin sections of the resulting small membrane-derived vesicles failed to reveal any morphological differences between vesicles formed in the absence and presence of cytochalasin B.

DISCUSSION

We have chosen to investigate the action of cytochalasin B on the facilitated diffusion of glucose through human erythrocyte membranes because, although these cells have both microfilaments[14] and an unusually rapid hexose transport system, they undergo no known energy-requiring cytokinetic events; furthermore, erythrocyte membranes are easily prepared, and glucose translocation may be conveniently and accurately measured. Previous studies with this compound have been carried out with cells such as cultured chick fibroblasts[10] and cultured tumor cells[8] which actively metabolize glucose, and undergo energy-requiring movements of various types. Their active metabolism has necessitated the use of non-metabolizable glucose analogues such as 2-deoxyglucose and 3-*O*-methyl glucose, which are trapped intracellularly by phosphorylation, while the rate of glucose transport through human erythrocyte membranes is so rapid by comparison with phosphorylation that the latter can essentially be ignored.

Using two independent methods for studying glucose translocation through erythrocyte membranes, we have clearly shown that cytochalasin B is a remarkably potent inhibitor of this process. It is well established that the glucose transport system of erythrocyte membranes is symmetrical[21], since the maximal rates of translocation and the half-saturation constants for glucose are identical for glucose permeation of the membrane from either the inner or outer surface. The methods which we have employed have measured the effect of externally added cytochalasin B upon efflux of glucose from the erythrocyte and influx of glucose into erythrocyte ghosts. Essentially identical results were obtained. In both cases, cytochalasin B acted as a noncompetitive inhibitor having a K_i of 0.34 and 0.70 μM. It should be noted that, although the K_i for cytochalasin is almost identical for human erythrocytes and for cultured chick fibroblast cells, the data of Kletzien and Perdue[10] indicate that cytochalasin B is a competitive inhibitor of hexose translocation in the latter cells. The reason for this difference is not apparent at the present time. Since the accumulation of sugars in fibroblasts is a relatively complicated process in which the permanent isotopically labeled hexose is predominately phosphorylated and thus trapped, but the free sugar is capable of bidirectional diffusion, the actual kinetic analysis of translocation becomes difficult indeed. The methods which we have employed with human erythrocytes avoid these difficulties and allow much simpler and more precise kinetic analysis. Thus the differences between our results and those of Kletzien and Perdue[10] may be more apparent than real. On the other hand, it is possible that the mode of action of cytochalasin B is different in these two cell types, even though the close similarity of the inhibition constants renders this unlikely.

It is of interest that cytochalasin B, a noncompetitive inhibitor of glucose transport through the erythrocyte membrane, is bound to a finite number of sites, approx. 330 000 per erythrocyte, which is approximately equal to the number of glucose transport sites estimated by others[22] to be present in the erythrocyte membrane. In contrast, it has been reported that phloretin, wich appears from kinetic studies to be a competitive inhibitor of glucose transport in erythrocytes[23], is simply partitioned between the membrane and the surrounding fluid and has no fixed number of binding sites. Whether cytochalasin B is bound to a component

of the glucose transport system or exerts its inhibitory effect by interaction with some other membrane component which does not directly participate in hexose translocation cannot be decided from the present information. However, Mizel and Wilson[9] have demonstrated that although cytochalasin B is a potent inhibitor of glucose entry into HeLa cells, it has no effect upon transport of Ca^{2+}, thymidine, uridine, or α-amino-isobutyrate even when added at concentrations as high as 3.1 μM. Although similar studies have not been carried out with the human erythrocyte, the available evidence suggests that cytochalasin *B* rather specifically inhibits monosaccharide transport. Furthermore, we have observed no alteration in the morphology of erythrocyte membranes treated with cytochalasin B at concentrations which completely inhibit glucose transport, which suggests that the agent does not act by disrupting membrane morphology and function in any gross, non-specific manner. The evidence, although not compelling, suggests that cytochalasin B may be interacting with the hexose transport site. It is of interest in this connection that the cytochalasin B binding sites are approximately equal in number to the numbers of each of five major classes of proteins present in the human erythrocyte membrane. Fairbanks *et al.*[24] have found five major protein classes and one major glycoprotein which are separable by sodium dodecyl sulfate–acrylamide gel electrophoresis of sodium dodecyl sulfate-solubilized human erythrocyte membranes. They have estimated that the membrane of each erythrocyte contains approx. 260 000 glycoprotein molecules, and between 340 000 and 460 000 molecules of each of four of the five major protein bands. Although each electrophoretically separated protein band may contain more than one molecular species, it is apparent that a close similarity exists between the number of cytochalasin binding sites and the number of protein molecules of several classes present in the membrane. The Scatchard-type plots of the data we have obtained for the equilibrium binding experiments are linear with no suggestion of more than one type of binding site. It is therefore a distinct possibility that one of the electrophoretically distinguishable proteins in fact contributes the cytochalasin B binding site. More work will obviously be necessary to test this possibility.

As previously mentioned, cytochalasin B inhibits glucose transport identically whether added to the side of the membrane *cis* or *trans* with respect to glucose. In this it resembles phloretin[23] and stilbestrol[25]. Whether this reflects penetration of the membrane by cytochalasin B, interaction with a mobile carrier which moves freely between the two surfaces, or interaction with a molecule such as the major protein which spans the erythrocyte membrane[26] remains to be elucidated.

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