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CYTOCHALASIN B INHIBITION OF BRAIN GLUCOSE TRANSPORT AND THE INFLUENCE OF BLOOD COMPONENTS ON INHIBITOR CONCENTRATION

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Summary

The effect of cytochalasin B on cerebral glucose transport and metabolism was investigated in 19 isolated perfused dog brain preparations. Cytochalasin B is a potent, non-competitive inhibitor of glucose transport at the blood-brain interface. Both glucose transport into (K_i = 6.6 ± 1.9 μM) and out of the capillary endothelial cell are inhibited. The inhibition is readily reversible by perfusion with blood containing no cytochalasin B. After 2 min of exposure to 30 μM cytochalasin B, the cerebral oxygen consumption decreased by 31% probably due to decreased availability of glucose for oxidative metabolism. About one-half of the cytochalasin B that is dissolved in blood is bound to erythrocytes and other blood components while the remainder is free.

Introduction

Cytochalasin B, a fungal metabolite, inhibits hexose transport in a wide variety of cell types, including erythrocytes [1], leukocytes [2], adipose cell ghosts [3], fibroblasts, liver cells [4] and HeLa cells [5].

In the nervous system, cytochalasin B inhibits the uptake of 2-deoxy-[^{3}H]-glucose by frog dorsal ganglia cell bodies [6] and uptake of 3-*O*-methyl-D-glucose by cultured human glioma cells [7]. Uptake of amines into peripheral adrenergic neurones is also inhibited by cytochalasin B under anaerobic conditions because there is insufficient glucose for glycolytic metabolism [8,9]. Glucose transport is of fundamental importance to the nervous system since,

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under normal conditions, glucose is the only metabolite taken up from the blood stream in sufficient quantities to maintain cerebral function. It is therefore surprising that cytochalasin B inhibition of hexose transport in the central nervous system has not been studied more extensively.

We have used the isolated perfused dog brain preparation to study the effects of cytochalasin B on the kinetics of unidirectional glucose transport into the brain. The preparation is valuable for the study of glucose transport in the nervous system since the relationship between anatomic structures is maintained together with other characteristics of an in vivo preparation. It also has the advantage of an in vitro preparation because the influence of other tissues is eliminated and physiologic and metabolic parameters are controlled.

Methods

Nineteen adult mongrel dogs were used in this study. Details of the preparation and maintenance of the isolated perfused dog brain have been published previously [10,11]. The extracranial soft tissues are removed, the spinal cord transected at the level of the second cervical vertebra and the brain, enclosed within the cranium, is perfused with compatible donor blood through the internal carotid arteries and the anastomotic branch of the internal maxillary arteries. The venous blood is collected through an outlet threaded into the confluence of venous sinuses. The perfusion apparatus [12] consists of two separate pump oxygenator systems interconnected through a valve which permits perfusion from only one oxygenator at a time. However, perfusion can be changed from one oxygenator to the other without any change in perfusion pressure or blood flow rate. The perfusate consisted of compatible, conditioned, donor blood, diluted in vivo with dextran-40 to a hematocrit of 25—30%. Blood with low glucose concentration was obtained from donor dogs that received 10—20 international units of insulin at the time of hemodilution. Under these conditions increased insulin levels have no effect on unidirectional influx of glucose into the canine brain [13]. Both oxygenators were filled with identical blood and equilibrated with a mixture of air and CO_2 to give a P_{O_2} of greater than 100 mm Hg and a P_{CO_2} of 37—42 mm Hg. Arterial pH averaged 7.38. The glucose concentration of the control oxygenator was maintained between 4.5 and 6 mM. The preparation was perfused from this oxygenator except for brief periods (2—3 min each) when it was perfused with blood from the experimental oxygenator. The blood in the experimental oxygenator contained a fixed concentration of cytochalasin B which was dissolved in absolute ethanol before addition to the perfusate. The alcohol concentration never exceeded 1 ml/l blood. To study the influx of glucose over a range of blood glucose concentrations appropriate for kinetic analysis, the glucose concentration of the experimental oxygenator was increased step-wise from 1 to 64 mM.

The indicator dilution technique [13] was used to measure the unidirectional glucose influx at the various glucose and inhibitor concentrations. 15 s before each determination, the valve was switched so that the brain was perfused from the experimental oxygenator, and 50 μl of a solution containing 2 μCi of ^{22}Na and 10 μCi of D-[6-^{3}H]glucose diluted in saline was rapidly injected into a port in the common carotid artery near the internal carotid

bifurcation. 30 consecutive venous blood samples were collected at 1-s intervals and subsequently digested and decolorized for simultaneous liquid scintillation counting of ^{22}Na and ^{3}H as previously described [13]. Arterial and venous blood samples were simultaneously collected for analysis of glucose and oxygen. Blood flow was determined by measuring the volume of a 1 min collection of venous blood. The valve was returned to the initial position so that the brain was again perfused from the control oxygenator. Plasma glucose concentrations were determined in triplicate by a glucose oxidase method using a Beckman glucose analyzer. Whole blood oxygen concentrations were determined from the output of a fuel cell (Lex-O_2-Con, Lexington Instrument Corp., Waltham, Mass.). Cytochalasin B was obtained from Aldrich Chemical Co. [^{3}H]Cytochalasin B was purchased from New England Nuclear.

The reversibility of cytochalasin B inhibition was studied in three brain preparations. In each preparation the control glucose influx was determined with the brain perfused from the control oxygenator. The brain was then perfused from the experimental oxygenator containing 30 μM cytochalasin B. After 60—70 s, perfusion from the control oxygenator was resumed and unidirectional glucose influx determined 4 min later. The glucose concentrations in both oxygenators were maintained equal.

The viability of the preparation was assessed at frequent intervals from electroencephalographic recordings (EEG), cerebral oxygen consumption and cerebral vascular resistance (perfusion pressure/flow rate). At the end of each experiment, the brain was removed and weighed.

Calculations

The maximal extraction of glucose for each indicator dilution injection was determined as previously described [13]. The rate of unidirectional glucose influx, v, was calculated from the following equation

$$v = (E - 0.036)\,\bar{A}\,\frac{F_p}{W}$$

where E is the maximal extraction of glucose (0.036 is a correction for diffusion), $\bar{A}$ is the mean of the arterial and venous plasma glucose concentrations, F_p is the plasma flow rate, and W is the brain weight. Because the rate of unidirectional glucose influx varies with the plasma flow rate [13], only indicator dilution studies conducted at plasma flow rates between 0.44 and 0.56 ml/g of brain per min were included. Glucose influx as a function of plasma glucose concentration for control and each concentration of cytochalasin B was fitted to the Michaelis-Menten equation by a computer program [14] which employs an iterative, least-squares method.

To calculate the inhibition constant, K_i, the data at all concentrations of cytochalasin B were fitted by computer to the following equation which describes non-competitive inhibition:

$$v = \frac{V\bar{A}}{K_m\left(1 + \frac{I}{K_{is}}\right) + \bar{A}\left(1 + \frac{I}{K_{ii}}\right)} \qquad (1)$$

where K_{is} is the constant for dissociation of inhibitor from the carrier-inhibitor complex and K_{ii} is the constant for dissociation of inhibitor from the carrier-substrate-inhibitor complex. If $K_{is} = K_{ii}$, then Eqn. 1 describes simple linear non-competitive inhibition and a double reciprocal plot of $1/\bar{A}$ vs. $1/v$ will intercept the $1/\bar{A}$ axis at $-1/K_i$. However, if $K_{is} \neq K_{ii}$, then reciprocal plots will not intersect on the $1/\bar{A}$ axis indicating that either slope, intercept or both are varing as a function of inhibitor concentration. Such a variation may be observed graphically from a plot of slope or intercept versus inhibitor concentration.

Net glucose uptake by the brain is related to unidirectional glucose transport by the following equation:

$$\text{Net glucose uptake} = v_{in} - v_{out}$$

In this equation, v_{in} is the rate of glucose transport from blood to brain and v_{out} is the rate of glucose transport from brain to blood. By rearranging, the equation can be solved for v_{out} and glucose efflux can be calculated from net glucose uptake (arterio-venous differences) and v_{in} (indicator dilution method). It is assumed that unidirectional glucose efflux is a function of the glucose concentration in the brain. By perfusing the brain with blood containing a fixed glucose concentration (5—6 mM), the glucose concentration in the brain and, therefore, v_{out} is held constant.

Results

Glucose influx was determined in 19 perfused dog-brains. Cytochalasin B was present at 0, 5, 10, 20 or 30 μM and the experimental glucose concentration was varied from 1 to 64 mM. Double reciprocal plots ($1/v$ vs. $1/A$) for representative data at 0, 10 and 20 μM cytochalasin B are shown in Fig. 1. The combined data from control and all cytochalasin B-treated groups (Fig. 2) indicates that cytochalasin B causes an inhibition of glucose influx that is characterized by an increase in the intercept on the $1/v$ axis and an increase in the slope. The combined data of Fig. 2 (135 values) were fitted by com-

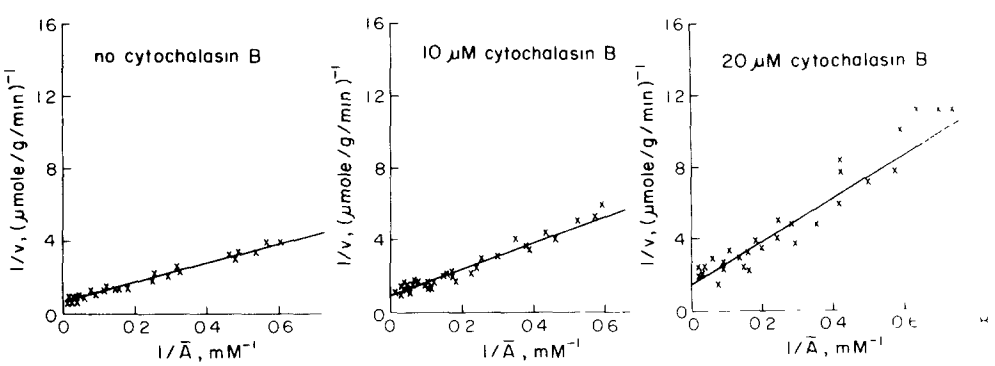

Fig. 1. Inhibition of glucose transport by cytochalasin B. Representative data of $1/\bar{A}$ is plotted versus $1/v$ at 0, 10 or 20 μM cytochalasin B (total). Each point represents one influx measurement. All lines and kinetic constants were derived from a least-squares fit of the corresponding data to the Michaelis-Menten equation with the aid of a computer.

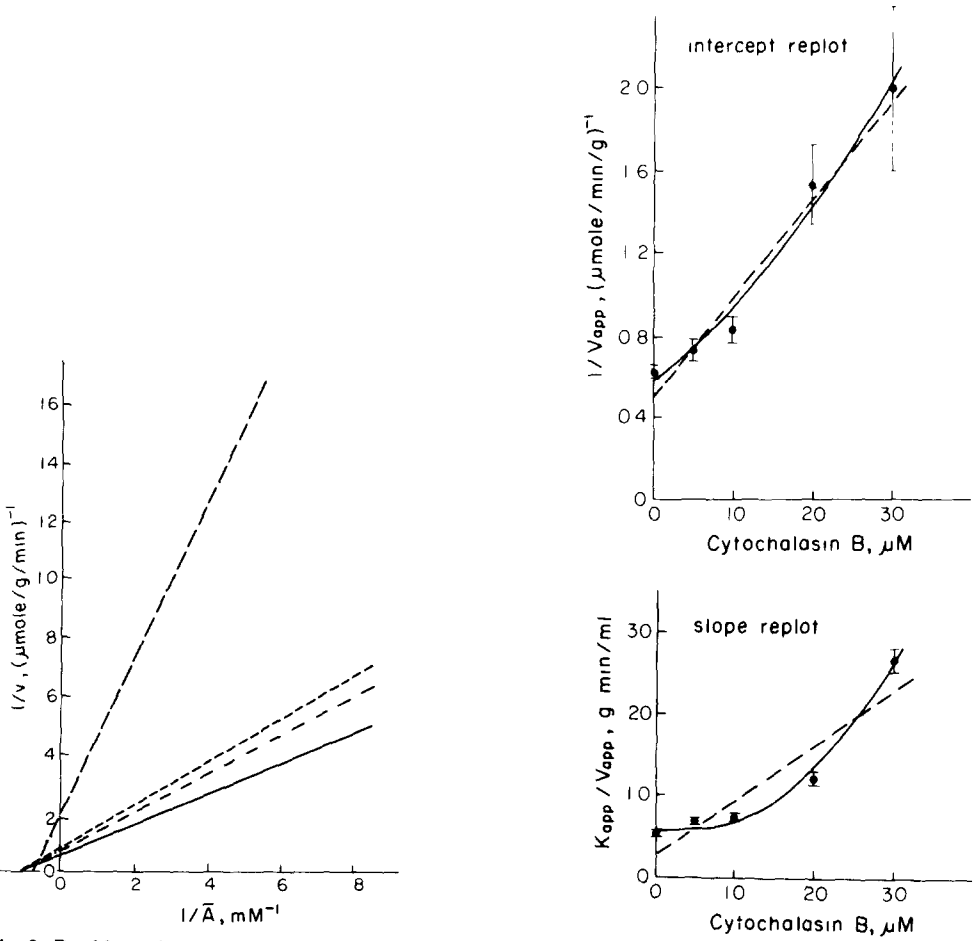

Fig. 2. Double reciprocal plot of glucose transport versus glucose concentration at a series of cytochalasin B concentrations. ———, no cytochalasin; — · — ·, 5 μM cytochalasin; - - - - - -, 10 μM cytochalasin; · · · · · ·, 20 μM cytochalasin and — — —, 30 μM cytochalasin. Individual data points have been omitted. Curves were obtained as in Fig. 1.

Fig. 3. (A) Replot of the slope (K/V, g/min per ml) derived from Fig. 2 at each cytochalasin B concentration (μM) versus the cytochalasin B (total) concentration. The broken line describes the data fitted to a linear function; the solid line represents the same data fitted to a hyperbolic function. K/V values are shown ±1 S.E. (B) Replots of the intercept ($1/V$, g/min per μmol) derived from Fig. 2 at each cytochalasin B concentration (μM) versus the cytochalasin B (total) concentration. The broken line shows the data fitted to a linear function; the solid line is the same data fitted to a hyperbolic function. $1/V$ values are shown ±1 S.E.

puter to Eqn. 1 yielding estimated values for K_{is} and K_{ii} of 12.3 ± 1.6 and 15.4 ± 4.1 μM, respectively. The combined data were also fitted by computer to the Michaelis-Menten equation, which describes competitive inhibition. However, the fit was totally unsatisfactory and resulted in the computation of extremely large standard errors. A replot of the intercept (Fig. 3A) versus the cytochalasin B concentration appears to be fitted equally well by either linear or hyperbolic functions. The slope replot (Fig. 3B) is more hyperbolic in nature, as a result of the decrease in the intercept on the $1/\bar{A}$ axis as the cyto-

chalasin B concentration becomes large. Therefore, the inhibition of glucose influx by cytochalasin B may be described as I-linear S-parabolic non-competitive inhibition.

The concentration of cytochalasin B used in plotting the kinetic data was the concentration added to the blood in the perfusion system. To determine if the apparent non-linerarity of the slope and intercept replots was due to the inhibitory mechanism or to the binding of cytochalasin B to blood elements, samples of perfusate (5 ml, hematocrit 30%) were dialyzed against five different concentrations of [^{3}H]cytochalasin B (95 ml volume, 0.9% saline containing 5.6 mM glucose for 48 h at 2°C). The radioactivity present in the perfusate and dialysate was analyzed and the concentration of cytochalasin B in the dialysate, termed unbound cytochalasin B, was calculated. The relationship between total and unbound cytochalasin B (Fig. 4) demonstrates that a portion of the cytochalasin B added to the perfusion system was bound to some unidentified blood components (perhaps red cells and plasma proteins) and hence was not available to inhibit brain glucose transport. The kinetic data were plotted (Fig. 5)

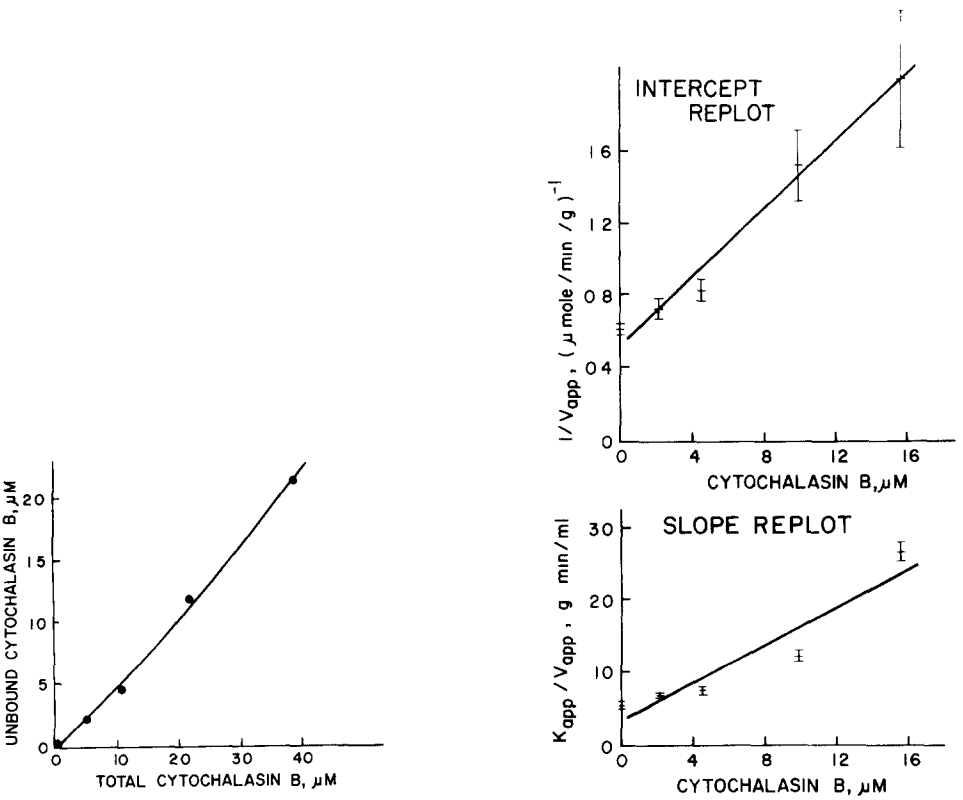

Fig. 4. The relationship between total and unbound cytochalasin B of perfusate. 5 ml of perfusate (hematocrit 30%) were dialysed against five different concentrations of [^{3}H]cytochalasin B (volume 95 ml, 0.9% saline containing 5.6 mmol/l glucose) for 48 h at 2°C. The concentration of cytochalasin B in the dialysate is termed unbound cytochalasin B.

Fig. 5. Replots of the intercept and slope at each cytochalasin B concentration against the unbound cytochalasin B concentration. Values are shown ±1 S.E.

TABLE I

THE REVERSIBILITY OF CYTOCHALASIN B INHIBITION

Control influx of glucose was determined with the brain perfused from the control oxygenator The brain was then perfused for 60—70 s from the experimental oxygenator containing 30 μM cytochalasin B. Perfusion was then resumed from the control oxygenator. A further determination of influx was made 4 min later F_p/W values were the same in both groups. There was no significant difference between the glucose influx of the controls and the corresponding values obtained following exposure to cytochalasin B.

	Blood glucose (μmol/ml ± S E.)	Glucose influx (μmol/g per min ± S E.)
Control (n = 3)	5.08 ± 0 36	0.582 ± 0.036
4 min after exposure to cytochalasin B (n = 8)	5.01 ± 0 29	0.535 ± 0 027

by substituting the unbound cytochalasin B concentration for the total cytochalasin B used in Fig. 3. Although there was still evidence of non-linearity in the slope replot, straight lines were fitted to both the intercept and slope replots. An average value of 6.6 ± 1.9 μM was obtained when K_i was recalculated.

The reversibility of cytochalasin B inhibition is shown in Table I. The effect of cytochalasin B was reversed within 4 min of the start of brain perfusion with cytochalasin-free blood. Other time intervals were not studied.

Exposure of the brain to the hexose transport inhibitor, cytochalasin B, did not alter the rate of oxygen consumption until the level of inhibitor in the perfusate reached 20 μM (Table II). A very large fall (31%) in oxygen consumption was observed when the cytochalasin B concentration was increased to 30 μM.

The efflux of glucose from the brain of control animals was 0.46 ± 0.04 μmol/g brain per min and was independent of blood glucose concentration between 0.8 and 25 mM (Table III). Inhibition of glucose efflux progressively increased as the cytochalasin B concentration increased, inhibition reaching 72% at 30 μM (Table III).

TABLE II

EFFECT OF CYTOCHALASIN B ON CEREBRAL OXYGEN CONSUMPTION

The brain was perfused for about 140 s from the experimental pump/oxygenator system which contained the glucose transport inhibitor. Arterial and venous blood samples were then collected for oxygen analysis.

Total cytochalasin B (μmol/l)	Oxygen consumption (ml/100 g per min ± S.E.)	
0	4.64 ± 0.15	(N = 31)
5	4.43 ± 0.24	(N = 9)
10	4.59 ± 0.16	(N = 18)
20	4.26 ± 0.11 *	(N = 16)
30	3.21 ± 0.05 **	(N = 15)

* $P < 0.1$.
** $P < 0.005$.

TABLE III

EFFECT OF CYTOCHALASIN B ON GLUCOSE EFFLUX FROM THE BRAIN

The calculation of glucose efflux is described in the text The range of glucose varied from 0 8 to 25 mM

	Total cytochalasin B concentration (μmol/l)				
	0	5	10	20	30
Unidirectional glucose efflux (μmol/g per min ± S E)	0.46 ± 0 04 (*N* = 25)	0 31 ± 0.04 * (*N* = 15)	0 26 ± 0 03 * (*N* = 31)	0 18 ± 0 02 * (*N* = 22)	0 13 ± 0 04 * (*N* = 15)

* $P < 0.01$

No abnormalities in the EEG pattern were observed during the short period that the brains were exposed to cytochalasin B. However, the presence of inhibitor appeared to ameliorate the effects of extreme hypoglycemia during indicator dilution experiments at very low glucose (1—2 mM) and high cytochalasin B (30 μM) concentrations. This observation may be due to the inhibition of glucose efflux and, therefore, the temporary maintenance of the cerebral glucose concentration during the 2—3 min hypoglycemic episode.

No significant alterations in cerebral vascular resistance were observed.

Discussion

Glucose transport into the capillary endothelial cell possesses the characteristics of a carrier-mediated, facilitated transport system [15—18]. Several structural analogs of glucose including 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose have a high affinity for the glucose carrier, compete with glucose for binding to the carrier and inhibit glucose transport in a competitive manner [18—20]. The present study demonstrates that inhibition of glucose transport into the brain by cytochalasin B differs from that caused by structural analogs in two important aspects. Cytochalasin B inhibition is non-competitive and, therefore, interacts at a location other than the site at which glucose and its analogs bind. The cytochalasin B binding site may be on the carrier or in close proximity to it. The inhibition by cytochalasin B is much more potent than that of the glucose analogs, the inhibition constant for cytochalasin B being an order of magnitude lower than those for the glucose analogs [18].

The slope of the intercept replot (Fig. 5B) is finite at low inhibitor concentrations. This suggests that the binding of one molecule of cytochalasin B to the carrier is enough to cause inhibition. There is, however, some evidence to suggest that the slope replot may be fitted by a hyperbolic function. This is still evident when the unbound cytochalasin B concentration is substituted for total cytochalasin concentration. The hyperbolic nature of the slope replot may point to a cooperative binding effect of additional cytochalasin molecules to the carrier of the form:

$$E + I \rightleftarrows EI + I \rightleftarrows EI_2 + I \rightleftarrows EI_3 + I \rightleftarrows EI_n$$

where each successive complex increases inhibition. This hypothesis, while tentative, suggests a direction for further study.

Since there are no previous kinetic studies of the action of cytochalasin B on brain glucose transport, comparisons must be restricted to studies of hexose transport in other cellular types. Such comparisons may be useful since several authors (reviewed by Betz et al. [21]) have pointed to basic mechanistic and structural similarities between hexose transport into the brain and across the erythrocyte membrane. There appear to be discrepancies in the literature regarding the nature of cytochalasin B inhibition of hexose transport across the erythrocyte membrane. Some authors report non-competitive inhibition [1,22] while others report competitive inhibition [23,24]. These discrepancies have been attributed to differences in techniques and experimental conditions.

The relationship between the total and unbound cytochalasin in our studies may be related to binding of cytochalasin B to erythrocytes, but binding to other blood components cannot be overlooked. There appear to be high and low affinity binding sites for cytochalasin B on the erythrocytes [23] and the presence of these sites complicates the interpretation of the present data. As the glucose concentration of the perfusate is increased, inhibition of cytochalasin B binding to high affinity sites on the erythrocyte membrane may increase the concentration of unbound cytochalasin available for binding to carrier sites in the brain. There is also the possibility that high and low affinity binding sites may exist in the brain. Further studies are necessary to differentiate between these alternatives.

A reduction in the supply of substrate will ultimately result in a decreased rate of oxidation. Therefore, the decrease in oxygen consumption during perfusion with high levels of cytochalasin B was predictable (Table II). No change in the rate of oxygen consumption, which would indicate a decrease in the glucose supply, was detected during perfusion for 2—3 min with 5 or 10 μM cytochalasin B. An alternative explanation for the reduced oxygen arteriovenous difference is that cytochalasin B affects the dissociation of oxygen and hemoglobin or release of oxygen by the erythrocyte in such a way that oxygen delivery is impaired. It is clear from the dialysis data (Fig. 4) that a major fraction of the cytochalasin B is bound to non-dialyzable blood components.

Cytochalasin B is an inhibitor of glucose transport out of the brain as well as glucose transport into the brain (Table III). This suggests that cytochalasin B either binds to the glucose efflux mechanism from the lumen side of the capillary endothelial cell or readily penetrates the cell and binds to the carrier from the inside. In either case, the transport system for glucose efflux is not distinguishable from the transport system for influx and, in fact, may be identical. If it is assumed that (1) glucose influx and efflux share the same carrier, (2) no cosubstrates are involved and (3) the transport mechanism is symmetrical, i.e. the dissociation constants for glucose binding are equal on the inside and outside of the membrane, then the glucose concentration of the compartment into which glucose is transported (probably the capillary cell) is calculated to be 3.4 mM. This value is obtained by solving the following Michaelis-Menten equation for S:

$$v_{out} = \frac{V \cdot S}{K_m + S}$$

where v_{out} = 0.46 μmol/g brain per min, V = 1.65 μmol/g brain per min and

K_m = 8.64 μmol/ml [20]. This value for the glucose concentration in the capillary endothelial cell suggests that glucose transport into the brain proceeds down a concentration gradient although it does not exclude the possibility that energy may be involved in the process [21]. Na^+ does not appear to be a cosubstrate in blood-brain glucose transport [25] as it is in some tissues.

It is clear that cytochalasin B is a very potent, readily reversible, non-competitive inhibitor of glucose transport at the blood brain interface and as such may provide a valuable experimental tool for further study of the metabolic and functional consequences of glucose transport inhibition into the brain.

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