Structure of Cytochalasins and Cytochalasin B Binding Sites in Human Erythrocyte Membranes†

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ABSTRACT: Twenty cytochalasins were tested for binding to and for inhibition of glucose transport in human erythrocyte membrane. In this membrane three cytochalasin B (CB) binding sites have been identified. All but three of the cytochalasins bind at site II. On the other hand, only nine of them, which are structurally closely related, bind at site I and inhibit glucose transport. For site I (and site III) binding and glucose transport inhibitory activities (a) the macrocyclic ring in the cytochalasin molecule must be at least 13-membered, (b) the nature of the aromatic ring at C-10 is not important, (c) the C-20–C-23 region makes a major contribution, and (d) the C-5–C-7 segment has a relatively minor influence. These findings do not support a proposed mechanism which involves 24, C-23, C-20, and C-1 oxygen atoms for interaction of CB with glucose carrier. The structural requirements for site II activity are less stringent. The size and the structure of the macrocyclic ring and the nature of the aromatic residue at C-10 modulate this activity only slightly, if at all. Modifications in the C-5–C-7 region of the molecule, however, result in substantial changes in this activity.

In human erythrocyte membrane, there exist three classes of CB[1] binding sites (sites I, II, and III) each of which shows distinct affinity and specificity (Jung & Rampal, 1977; Lin & Spudich, 1974). The binding at site I, the lowest affinity and largest capacity site, is competitively inhibited by glucose carrier substrates and inhibitors. Cytochalasin B and cytochalasin A competitively inhibit glucose carrier activity of the membranes, and the dissociation constant (K_D) for site I CB or CA complex is essentially identical with the inhibition constant (K_i) for CB or CA as carrier inhibitor. CB and CA also bind to other sites, site II and site III, but their dissociation constants do not correspond to the carrier inhibition constants. Site II is considered to be related to cell motility and cell morphology (Lin & Lin, 1978, 1979; Tannenbaum et al., 1977).

On the other hand, cytochalasins C, D, and E and some derivatives of CB, such as 21,22-dihydro-CB, neither bind at site I (and site III) nor inhibit glucose carrier activity. However, they readily bind to site II with a varying degree of relatively high affinities. Apparently, there is a specific structural requirement for site I binding which differs from that for site II binding. A study of the interaction of most of the 23 natural cytochalasins known at present (Natori, 1977) and some of the easily obtainable derivatives of cytochalasin B with human erythrocyte membrane was undertaken to delineate the exact structural requirements for site I and site II binding and glucose transport inhibitory activities. It was found that in addition to cytochalasins B and A, only seven cytochalasins, namely, 24-deoxaphomin, CB-7-monoacetate, chaetoglobosins B, E, and F, proxiphomin, and protophomin, show site I binding activity and at the same time inhibit glucose carrier activity, whereas all analogues except cytochalasin G and chaetoglobosins C and J show site II binding activity.

Experimental Procedures

Chemicals. Cytochalasins A, B, C, D, and E were obtained from Aldrich Chemical Co. [4-^{3}H]Cytochalasin B was purchased from New England Nuclear. 21,22-Dihydrocytochalasin B and CB-γ-lactone were generous gifts from Dr. D. C. Aldridge & ICI, England. Cytochalasin B 7,20-diacetate was prepared according to Rothweiler & Tamm (1970). Cytochalasin B 7-monoacetate was obtained according to the procedure of Masamune et al. (1977). 24-Deoxaphomin, protophomin, and proxiphomin were generously provided by Professor Ch. Tamm of the University of Basel, Switzerland. Cytochalasin G was a gift from Professor Fortes Cameron, University of Glasgow, U.K. Cytochalasin H was a gift from Dr. G. G. Christoph, The Ohio State University, Columbus, OH. Chaetoglobosins A, B, C, E, F, and J were very generously contributed by Professor S. Natori, National Institute of Hygienic Sciences, Tokyo, Japan.

Methods. Preparation of human erythrocyte membrane ghosts and measurements of glucose flux, binding of CB to the erythrocyte membranes, and displacement of bound CB by other cytochalasins have been described earlier (Jung & Rampal, 1977). Displacement of bound radioactive CB and inhibition of glucose transport by other cytochalasins are also briefly described in the figure legends. The apparent dissociation constants for binding of analogues at the binding site, K_D (K_I in the earlier paper), are derived from the displacement curves using the three-site model (Jung & Rampal, 1977) and knowing the relative binding at sites I, II, and III at 10^{-7} M CB concentration (see Results). The K_D for sites I and II for each displacing cytochalasin was assessed from the sensitivity of displacement to D-glucose and cytochalasin E, respectively.[2] A displacement of greater than 25% indicated the presence of site III, the K_D of which was not determined due to the additional complications when displacement was studied in the presence of D-glucose and cytochalasin E together (Pinkofsky et al., 1978).

Results

When freeze-dried ghosts were incubated (1 mg/mL) with 10^{-7} M radioactive cytochalasin B, ~76% of the radioactivity was bound to membranes (Figure 1a). Confirming our previous results, ca. 10, 15, and 70% of the bound radioactivity was associated with the sites I, II, and III, respectively, the remaining 5% representing nonspecific binding under these

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[1] Abbreviations used: CB, cytochalasin B; CA, cytochalasin A; BSS, balanced salt solution.

[2] A detailed account of this analysis will be published elsewhere.

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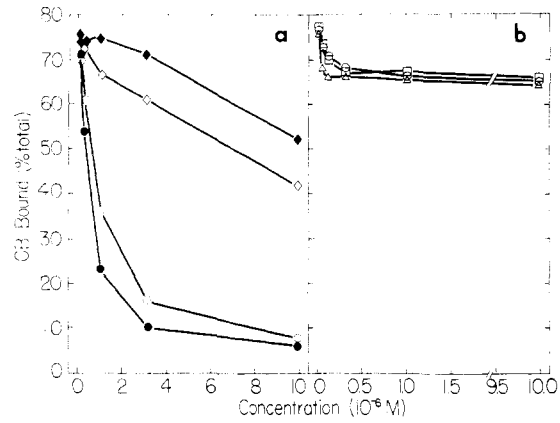


FIGURE 1: Displacement of radioactive cytochalasin B from erythrocyte membranes by different cytochalasins. Freeze-dried ghosts (1 mg/mL) were incubated in 1:10 BSS, pH 7.4, containing radioactive CB (0.04 μCi/mL; 3×10^{-7} M) for 30 min at 22 °C. A given cytochalasin (0.02 mL of appropriate stock solution in ethanol) was added to each 2-mL aliquot of this ghost suspension to give the specified final concentration ranging from 0 to 10^{-5} M. After a 30-min incubation at 22 °C, the aliquots were centrifuged (1500 rpm using the SS-34 rotor in a Sorvall RCB-2) for 30 min at 4 °C. The pellets and supernatants were assayed for their radioactivities by a liquid scintillation spectrometer (Nuclear Chicago, Mark II) using PCS (Amersham, Arlington Heights, IL) as the scintillation cocktail. Radioactivities of pellets as a percentage of the total radioactivity of the incubation mixture (pellet + supernatant) are plotted against overall concentrations of cytochalasins on the abscissa. Panel a (left): cytochalasin B (●), 24-deoxaphomin (○), proxiphomin (◊), and protophomin (♦) Panel b (right): cytochalasins E (○), C (□), and D (△).

Table I: Structure–Activity Relationships in Cytochalasins

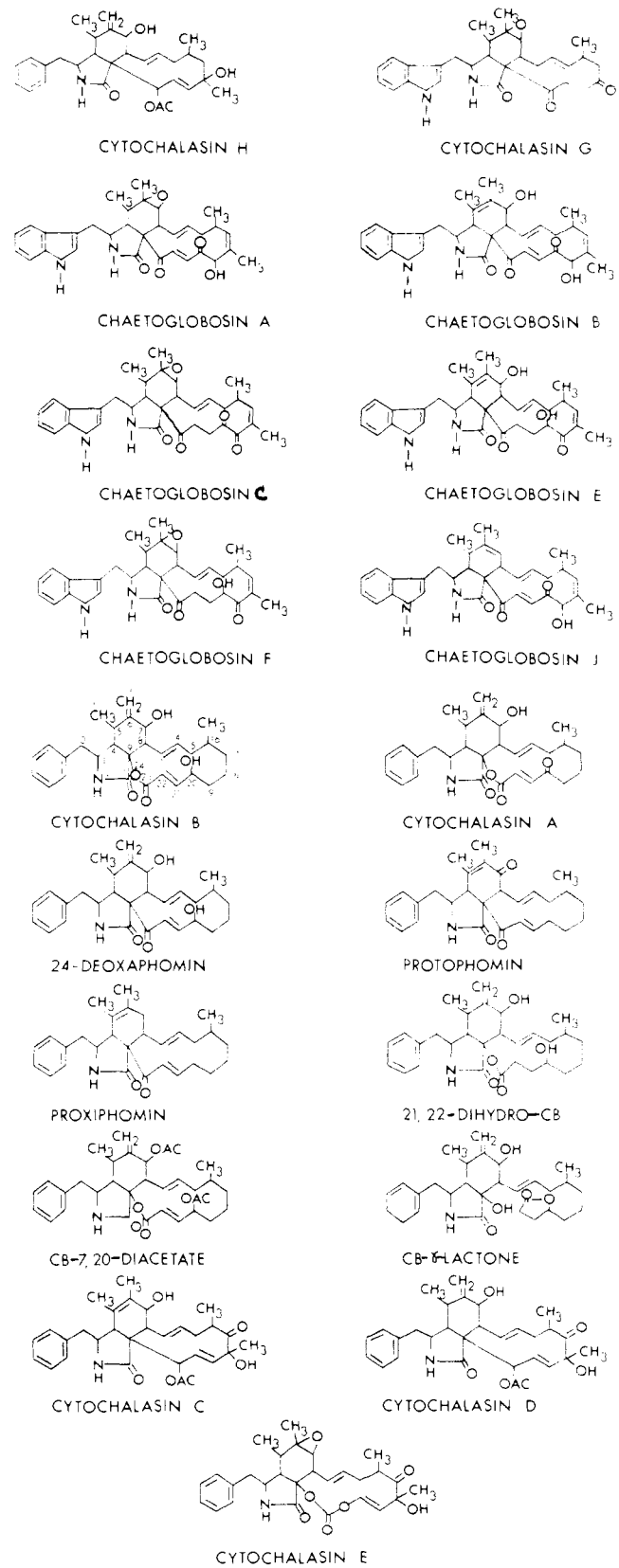
cytochalasin[a]	CB displacement, K_D site I (10^{-6} M)	site II (10^{-7} M)	site III[b]	carrier inhibn, K_i (10^{-6} M)
cytochalasin A	1.0–4.0	1.5	+	1.0–4.0
cytochalasin B	0.3–0.8	1.0–2.0	+	0.4–0.6
cytochalasin C	>100	0.8	–	>100
cytochalasin D	>100	0.2	–	>100
cytochalasin E	>100	0.9	–	>100
cytochalasin G	>100	>100	–	>100
cytochalasin H	>100	1.5	–	>100
CB-7-monoacetate	3.0	1.0	ND	2.0–4.0
CB-7,20-diacetate	>100	15–20	–	>100
21,22-dihydro-CB	>100	1.5	ND	>100
CB-γ-lactone	>100	2.0	ND	>100
24-deoxaphomin	1.2	1.0	+	1.0
proxiphomin	15	1.0	–	15
protophomin	15–18	≥15	ND	18
chaetoglobosin A	>100	1.0	–	>100
chaetoglobosin B	1.0–2.0	1.0	ND	10–20
chaetoglobosin C	>100	>100	–	>100
chaetoglobosin E	10–15	1.0	ND	10–15
chaetoglobosin F	15–20	1.0	ND	10–15
chaetoglobosin J	>100	>100	ND	ND

[a] For structures, see Chart I. [b] (ND) Not determined; (+) site III present; (–) site III absent.

experimental conditions (data not shown). Cold CB at an increasing concentration displaced almost all of the bound radioactivity, apparently affecting all three sites (Figure 1a), whereas cytochalasin E displaced not more than 15% of the bound radioactivity (Figure 1b), which is indicative of an interaction with site II binding only (Jung & Rampal, 1977).

Cytochalasin C or D, at an increasing concentration up to 10^{-5} M, displaced a maximum of ~15% of the bound radioactivity (Figure 1b), and this maximum displacement was unaffected by the copresence of an excess of cytochalasin E (data not shown). This would indicate that cytochalasins C and D, like E, interact with site II only. Similar results were obtained with cytochalasin H and chaetoglobosin A (not shown), which displaced cytochalasin B from only site II. The half-maximum displacement by these analogues occurred at concentrations which are significantly different, showing some affinity differential in their site II interaction (Table I and Chart I).

Chart I



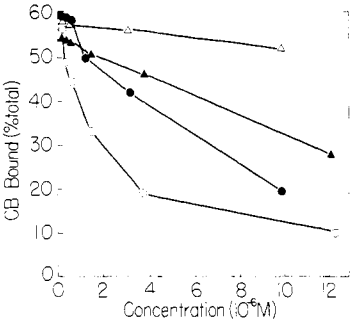


FIGURE 2: Displacement of radioactive cytochalasin B from erythrocyte membranes by cytochalasin B 7-acetate (●) and chaetoglobosins B (○), E (▲), and J (△). Experimental procedures and data presentation are otherwise the same as those of Figure 1.

In contrast, 24-deoxaphomin, proxiphomin, and protophomin each displaced the bound radioactivity by far more than 15%, and in this respect they are similar to cytochalasin B (Figure 1a). For example, 24-deoxaphomin displaced more than 90% of the bound cytochalasin B, indicating that all three sites are affected. A comparison of displacement curves indicated that the affinity to site I of 24-deoxaphomin is slightly (twofold) less than that of cytochalasin B and that their affinities to sites II and III are also not significantly different. An increasing concentration of proxiphomin up to 10^{-5} M displaced the bound radioactivity by 45%, with a tendency to increasing displacement at higher proxiphomin concentrations. The displacement–concentration profile suggested at least two saturable components: a high capacity–low affinity component and a low capacity–high affinity component. The lower affinity component was totally abolished in the presence of 1 M D-glucose (data not shown), thus identifying itself as site I. Protophomin at 10^{-5} M displaced the bound radioactivity by 30%, again with a tendency to increasing displacement at higher concentrations (Figure 1a).

Similar findings were obtained with cytochalasin B 7-monoacetate and chaetoglobosins B, D (Figure 2), and F (not shown), which indicated that these analogues also interact with all three sites. On the other hand, cytochalasin G (not shown) and chaetoglobosins C (not shown) and J displace CB only slightly from any of these sites (Figure 2). Estimates of the apparent binding affinities to site I and to site II for all of the cytochalasins tested are summarized in Table I, as obtained from the analyses of displacement experiments similar to these. Nine out of twenty cytochalasins tested show site I binding activity with the affinity sequence cytochalasin B > 24-deoxaphomin > chaetoglobosin B ≃ cytochalasin A > cytochalasin B 7-monoacetate > proxiphomin ≃ protophomin ≃ chaetoglobosin E ≃ chaetoglobosin F. All except three cytochalasins tested revealed site II binding activity. Cytochalasin D showed the highest affinity to site II whereas protophomin displayed the lowest affinity, with others showing intermediate affinities which are not too different from that of cytochalasin B.

Potencies of different cytochalasins as inhibitors of the glucose transport carrier in human erythrocytes are also included in Table I. Detailed aspects of the inhibition of this carrier mediation by cytochalasins B and A have been reported elsewhere (Jung & Rampal, 1977). Demonstrated in the present study is that 24-deoxaphomin, proxiphomin, protophomin (Figure 3), CB-7-acetate, and chaetoglobosins E, F, and B (Figure 4) also inhibit the glucose carrier activity. The carrier inhibition by each of these cytochalasins showed a saturable concentration dependency from which inhibition constants (K_i) (Table I) were obtained. Although some of the

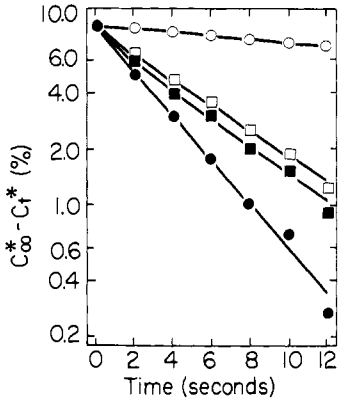


FIGURE 3: Time course of the isotopic equilibrium of D-mannose across erythrocyte membranes in the absence of any addition (●) and in the presence of 10^{-5} M 24-deoxaphomin (○), protophomin (■), and proxiphomin (□). Washed intact erythrocytes 2×10^{10} cells) were suspended in 150 mM NaCl, pH 7.4, buffered with 10 mM Tris-HCl containing 20 mM D-mannose in a final volume of 20 mL and incubated at 37 °C for 30 min. 0.2 mL of 10^{-3} M cytochalasin stock solution was added, and the mixture was equilibrated at 23.5 °C for another 30 min. At $t = 0$, an aliquot (0.36 mL) of D-[^{14}C]mannose solution (20 mM, 3.6 μCi) was quickly added to the suspension and a series of six 2-mL aliquots were taken after every 2 s. Each aliquot was shot into 8 mL of prechilled 150 mM NaCl buffer solution containing 2 mM $HgCl_2$. The ghosts were separated as pellets from the supernatant by centrifugation at 4 °C. Radioactivities of both pellets and supernatants were assayed and expressed as a percentage of the total radioactivity of the flux system. C_∞^* and C_t^* denote the radioactivities of pellets at complete tracer equilibration (measured at $t = 5$ min) and at a given time t, respectively. *Straight lines* represent a linear regression of the data; the temperature was at 20 °C.

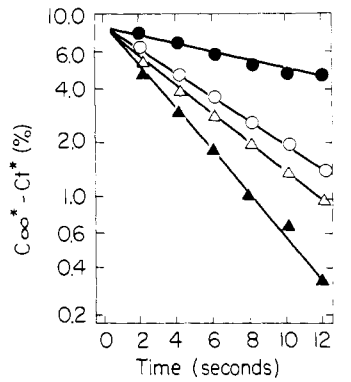


FIGURE 4: Time course of the isotopic equilibrium exchange of D-mannose across erythrocyte membranes in the absence of any addition (▲) and in the presence of 10^{-5} M cytochalasin B 7-monoacetate (●), chaetoglobosin B (△), and chaetoglobosin E (○). Experimental procedures and data presentation are otherwise the same as those of Figure 3.

values listed represent results of a single determination because of limited availability of the compounds, the correlation between K_i for transport inhibition and K_D for site I binding is impressive. The only exception is with chaetoglobosin B. This analogue, with its substantial site I activity, is expected to be a strong inhibitor of the glucose carrier activity. However, this correlation seems to break down in this case. This experiment could not be repeated due to the scarcity of the compound.

Discussion

Effect of the Macrocyclic Ring Size. Site I binding and glucose carrier inhibitory activities of cytochalasins show a strong correlation to the number of atoms in the macrocycle of the molecule. Cytochalasin B, a [14]cytochalasin, exhibits site I, site II, and site III binding activities and glucose carrier

inhibitory activity. Cytochalasin A, also a member of this group, shows all the above activities, although it is less potent in binding at site I and in inhibition of glucose carrier activity.

[13]Cytochalasins. Proxiphomin, protophomin, and 24-deoxaphomin belong to this group and show both site I binding and the glucose carrier inhibitory activities. The magnitude of these activities, however, is lower than that of CB. Chaetoglobosins also have a 13-membered macrocycle. Chaetoglobosins B, E, and F show site I binding activity to a varying extent; chaetoglobosins B, E, and F also inhibit glucose carrier activity. Chaetoglobosin A shows only site II binding activity, while chaetoglobosins C and J are totally nonactive. Cytochalasin E, also a [13]cytochalasin, shows only the site II binding activity.

[11]Cytochalasins. Cytochalasins C, D, and H, members of this group, show only the site II binding activity. Cytochalasin G, also a member of this group, is totally devoid of any activity. It appears that a minimum of a 13-membered ring in cytochalasin is required for site I binding and glucose carrier inhibitory activities. It may not, however, be the optimum size of the ring for these activities; CB, a [14]cytochalasin, when compared with 24-deoxaphomin, has higher site I binding activity. The size of the macrocycle, however, does not seem to be important to manifestation of site II binding activity and even its integrity is not essential as CB-γ-lactone in which the macrocyclic ring is open is site II active.

Effect of Variations in the Structure of the Macrocyclic Ring. It appears from the data in Table I that the C-20–C-23 region, namely, the C-20 hydroxyl, the C-21(22) double bond, and the C-23 carbonyl, plays a major role for the manifestation of site I binding activity. Absence of the 24-oxygen in the 24-deoxaphomin only slightly decreases the activity. The lactonic oxygen makes only a minor contribution to this activity in CB, or the small decrease in activity noted could be only a ring-size effect discussed earlier. Oxidation of the 20-hydroxyl to a ketonic function in CA decreases site I binding activity. Acetylation of both 7- and 20-hydroxyl groups abolishes this activity altogether. Cytochalasin B 7-monoacetate, however, has site I binding activity and inhibits glucose transport. Proxiphomin and protophomin, which lack the C-20 oxygen function, are less, but still, site I active. The lower affinity compared to that of 24-deoxaphomin could be due to the absence of the C-20 oxygen function. Saturation of the C-21(22) double bond in CB leading to 21,22-dihydro-CB results in total loss of site I binding activity. Chaetoglobosins E and F, which do not have a C-21(22) double bond, on the other hand, are still site I active. All the above compounds show site II binding activity. Cytochalasins C, D, E, G, and H, the site I inactive compounds, are grossly different in structure in this part of the molecule; they lack the C-20 oxygen function, the C-21(22) double bond, and the C-23 carbonyl function.

Chaetoglobosins carry an oxygen function at C-19, a double bond at C-17, and a methyl group at C-18 in addition to the C-20 oxygen function, the C-21(22) double bond, and the C-23 carbonyl group. Structure–activity relationships in chaetoglobosins appear to be much more complex. As indicated above, at least in chaetoglobosins the C-21(22) double bond is not essential for site I binding activity. Both chaetoglobosins E and F have a free C-20 hydroxyl group and C-19 and C-23 ketonic groups. In contrast, chaetoglobosin B, which has much higher site I affinity than chaetoglobosins E and F, has a C-20 keto group in addition to the C-23 carbonyl, the C-19 hydroxyl, and the C-21(22) double bond. Chaetoglogosins A and J are constituted in the same fashion as chaetoglobosin B in the C-19–C-23 region. However, chaetoglobosin A has no site I activity, and chaetoglobosin J is both site I and II inactive. Chaetoglobosin C is a C-19, C-20, C-23 triketone and is without site I and site II activities.

Taylor & Gagneja (1975) have proposed that the C-1, 24, C-23, and C-20 oxygen atoms in CB are involved in hydrogen bonding to glucose carrier protein and that 21,22-dihydrocytochalasin B does not show site I binding activity because the C-20 hydroxyl group in this compound cannot maintain the rigid position, obtained in CB, which is required for the binding to the carrier site. Contrary to the prediction from this model, 24-deoxahomin, which lacks the 24-oxygen atom, is a strong inhibitor of glucose carrier and has a strong affinity for site I. Furthermore, protophomin and proxiphomin, which are devoid of both the 24 and the C-20 oxygen atoms, are still substantially active.

Cytochalasins C, D, E, and H carry a methyl and a tertiary hydroxyl at C-18 and, except cytochalasin H, a carbonyl function at C-17. It is interesting to compare site II binding affinities of cytochalasin D ($K_D = 2 \times 10^{-8}$ M) and cytochalasin H ($K_D = 15 \times 10^{-8}$ M). Cytochalasin H differs from cytochalasin D only in not having a carbonyl at C-17 and having an opposite configuration at C-18.

Effect of Structure in the C-5–C-6–C-7 Region. This region of the molecule influences the site I activity in a relatively minor way. The structures of protophomin and proxiphomin in the C-5–C-6–C-7 region are different from those of CB and 24-deoxaphomin. In protophomin, the C-7 hydroxyl is oxidized to a keto group and the C-6(12) double bond is shifted to the C-5(6) position, while proxiphomin does not have a C-7 oxygen function and the C-6(12) double bond is isomerized to the C-6(7) position. Notwithstanding this gross difference in their structures in the C-5–C-6–C-7 region, protophomin and proxiphomin have equal site I binding activities. However, site I affinity for CB-7-monoacetate is 0.1–0.25 that of CB. On the other hand, for site II binding activity this region relative to other regions of the molecule could play a more important role. The differences in binding (K_D) of cytochalasins C, D, and E to site II could be correlated to the differences in their structures in the C-5–C-6–C-7 region. Cytochalasin D has a C-7 hydroxyl and a C-6(12) double bond and binds most effectively ($K_D = 2 \times 10^{-8}$ M) of the three. Cytochalasin C has a hydroxyl at C-7 and a C-5(6) double bond and binds less effectively ($K_D = 6 \times 10^{-8}$ M), while cytochalasin E, in which the C-7 hydroxyl and the double bond are isomerized to a 6,7-epoxide, binds least effectively ($K_D = 9 \times 10^{-8}$ M) of the three compounds. Lin & Lin (1978), however, report that relative affinities for site II in human erythrocytes are cytochalasin E > cytochalasin D > dihydrocytochalasin B > cytochalasin B.

Again, there is no clear-cut pattern in the case of chaetoglobosins. Chaetoglobosins E and F have similar affinities for site I and differ from each other only in the C-5–C-7 region, indicating that this region has little effect on site I binding activity. Chaetoglobosins A, B, and J also differ from one another only in the C-5–C-7 region; however, their site I and site II binding activities are grossly different. Chaetoglobosin B is site I active and has a C-5(6) double bond and a C-7 hydroxyl group. Chaetoglobosin A is site I inactive and has a 6,7-epoxide structure, while chaetoglobosin J does not have the C-7 oxygen function, has the C-6(7) double bond, and is without site I and II activities. Furthermore, chaetoglobosins A, C, and F all have the 6,7-epoxide structure but differ widely in their site I and II binding activities; chaetoglobosin F is site I active, chaetoglobosin A is site I inactive, and chaetoglobosin

C is both site I and site II inactive. On the other hand, chaetoglobosins B and E also have the similar C-5(6) en-7-ol structure and both are site I active, although they exhibit affinity differential. Chaetoglobosins A, B, E, and F have similar site II affinities.

Effect of Substitution at C-10. All cytochalasins, except cytochalasin G, carry a phenyl group at C-10. Chaetoglobosins and cytochalasin G have an indole group at C-10. The activities do not seem to be associated with the presence of any of the two groups in particular. Evidently, the nature of the aromatic group at C-10 has only a minor, if at all any, influence on manifestation of activities.

Glucose Carrier Inhibition and Site I Binding Activity. Based on close correlation between site I binding and glucose transport inhibition by several cytochalasins including cytochalasins B and E, we have previously concluded that site I binding protein is, or is closely related to, glucose carrier in human erythrocytes (Jung & Rampal, 1977). The results in the present study provide further support to this conclusion by establishing a very close correlation between the site I binding activity and the transport inhibition. Only an apparent exception is chaetoglobosin B, which showed some quantitative disagreement between its site I binding constant (K_D) and carrier inhibitor constant (K_i) (Table I).

Cytotoxic Effect and Structure of Cytochalasins. Minato et al. (1973) have examined a large number of derivatives of cytochalasin D in relation to their cytotoxic (in HeLa cells) and antitumor (in mice) activities. According to their study, a free hydroxyl group at C-7 is essential to manifestation of the activity. Reduction of the phenyl group to a cyclohexane derivative decreases cytotoxicity drastically. The C-17 carbonyl, the C-18 hydroxyl, and the double bonds in the macrocyclic and the cyclohexane rings are not essential for the activity; the integrity of the macrocycle, however, is. Furthermore, the position of the double bond in the C-5–C-6–C-7 region is not found to be important. Natori (1977), however, found that the nature of the aromatic residue at C-10 has no influence on cytotoxic behavior. Beno et al. (1977) considered that the invariance of the conformation of the core of the molecule and the direct substituents on the perhydroisoindole nucleus may be the most important factors in manifestation of cytotoxicity. The 1-CO, 2-NH, and C-7 OH functions are the principle rigidly configured binding groups. The macrocycle seems to be important for its bulk only.

It is apparent from the above findings that the structural requirements of cytotoxicity and antitumor activity are entirely different from those reported in the present investigation for site I activity, which exhibits a very narrow specificity. The structure–site II activity pattern, on the other hand, is much less but still substantially different from the structural requirements for the cytotoxic effect of cytochalasin D derivatives. In contrast to the cytotoxic effect in cytochalasin D derivatives, for site II activity the C-5–C-6–C-7 region is important but the presence of a free C-7 hydroxyl group is not obligatory [site II active (also toxic) cytochalasin E does not have a free C-7 hydroxyl]. Cytochalasin B γ-lactone, in which the macrocycle is ruptured, still shows site II activity.

It may be emphasized, however, that entirely different cell lines are used in these studies and that different cell types show slightly different "site II" specificities. For example, in purified peripheral lymphocytes and polymorphonuclear leukocytes (Parker et al., 1976; Jung and Mookerjee, unpublished experiments) the glucose-insensitive component of CB binding is inhibited by cytochalasins in the order E > A > D > B. This order of inhibitory powers is slightly different from the site II affinities in human erythrocytes of these cytochalasins. Further, 21,22-dihydro-CB and CB-γ-lactone, which have substantial site II affinities in human erythrocytes, were found to be very weak inhibitors of CB binding to lymphocytes and polymorphonuclear leukocytes. Cytotoxicity, moreover, is a complex biological effect.

Finally, it may be mentioned that the relative potencies of cytochalasins D and E and dihydro-CB in bringing about morphological changes in mammalian fibroblasts (3T3 cells) were found to be parallel to the affinities of these cytochalasins (Lin's order) for site II (Lin & Lin, 1978).

Acknowledgments

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