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Location of Amino Acid and Carbohydrate Transport Sites in the Surface Membrane of Normal and Transformed Mammalian Cells

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Summary. Amino acid and carbohydrate transport in normal and malignant transformed hamster cells was studied after binding of the protein Concanavalin A (Con. A) to the surface membrane. Experimental conditions were used so that a similar number of Con. A molecules were bound to both types of cells. The transport of amino acids was inhibited after Con. A binding in the transformed cells but not in normal cells. This was found with the metabolizable amino acids L-leucine, L-arginine, L-glutamic acid, and L-glutamine, and with the non-metabolizable amino acids cycloleucine and α-aminoisobutyric acid. Transport of D-glucose and D-galactose was more inhibited by Con. A in transformed than in normal cells, and in both types of cells D-glucose was inhibited more than D-galactose. The inhibition by Con. A on transport was specific, since there was no effect on the transport of L-fucose in either normal or transformed cells. Con. A also did not effect the entry of 3-0-methyl-D-glucose.

These observations can be used to locate amino acid and carbohydrate transport sites in the surface membrane in relation to the binding sites for Con. A. The results indicate that Con. A sites are associated in normal cells with transport sites for D-glucose and to a lesser extent D-galactose, and in transformed cells with transport sites for amino acids and to a greater extent than in normal cells with D-glucose and D-galactose. Malignant transformation of normal cells therefore results in a change in the location of amino acid and carbohydrate transport sites in the surface membrane in relation to the binding sites for Con. A.

Studies with the carbohydrate-binding protein Concanavalin A (Con. A) (Summer & Howell, 1936), which binds to sites on the surface membrane, have shown a difference in the structure of the surface membrane between normal cells and malignant transformed cells. A variety of hereditarily transformed cells can be agglutinated by Con. A, but this protein only agglutinates normal cells after they have been treated with trypsin (Inbar & Sachs, 1969*a*). This agglutination was reversed by competition

with α-methyl-D-glucopyranoside (α-MG) and other carbohydrates that strongly bind to Con. A (Goldstein, Hollerman & Smith, 1965). Agglutinability by Con. A has been used to measure this structural change of the surface membrane also in abortively transformed cells (Inbar & Sachs, 1969*a*; Ben-Bassat, Inbar & Sachs, 1970). It has been shown that the difference in the structure of the surface membrane can be reflected by a differential toxic effect of Con. A on normal and transformed cells *in vitro*, and that local treatment of transplanted tumors with Con. A results in an inhibition of tumor development *in vivo* (Shoham, Inbar & Sachs, 1970).

The change in cellular regulatory mechanism that is produced by transformation can be ascribed to a change in the cell surface (Sachs, 1965; Hakomori, Teather & Andrews, 1968; Inbar & Sachs, 1969*a*, *b*; Burger, 1969; Sela, Lis, Sharon & Sachs, 1970). Direct evidence for this assumption for the α-MG-like sites was obtained by measuring the binding of Con. A molecules whose metal sites required for α-MG binding were labeled with ^{63}Ni. (Inbar & Sachs, 1969*b*.) The results indicate that malignant transformation of normal cells can be explained by three types of changes in the structural organizations of sites for Con. A in the surface membrane. There can be an exposure of cryptic sites, a concentration of exposed sites by a decrease in cell size, and a rearrangement of exposed sites without a decrease in cell size resulting in a clustering of sites (Ben-Bassat, Inbar & Sachs, 1971).

Mammalian cells can transport amino acids (Eagle, Piez & Levy, 1961) and carbohydrates (Alvarado, 1967) by an active transport mechanism. The membrane change in cell transformation seems to be accompanied by a change in the rate of transport of some but not all amino acids. Mouse 3T3 cells transformed by polyoma virus transported α-aminoisobutyric acid, cycloleucine, and L-glutamine more rapidly than untransformed 3T3 cells, whereas L-glutamic acid and L-arginine were transported as rapidly by untransformed 3T3 cells as by polyoma-transformed 3T3 cells (Foster & Pardee, 1969). Cell transformation by Rous sarcoma virus was associated with an increase in the transport of glucose, 2-deoxy-D-glucose, and glucosamine (Hatanaka & Hanafusa, 1970).

The present experiments were undertaken for two reasons: (1) to determine if the change in the structure of the surface membrane associated with malignant cell transformation can result in a differential effect by Con. A on the transport of amino acids and carbohydrates in normal and transformed cells; and (2) to determine the location of amino acid and carbohydrate transport sites in the surface membrane in relation to the binding sites for Con. A.

Materials and Methods

Cell Cultures

The transformed cells used in the present experiments consisted of lines of golden hamster cells transformed *in vitro* by polyoma virus, or after treatment with the chemical carcinogen dimethylnitrosamine (DMNA) (Huberman, Salzberg & Sachs, 1968), a line derived from a simian virus 40 (SV40)-induced hamster tumor, rat cells transformed by polyoma virus, and SWR mouse cells transformed by SV40. The normal cells used consisted of secondary cultures of golden hamster, rat, and mouse (SWR) embryo cells. Cells were grown in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 10% fetal calf serum in 50 mm plastic Petri dishes (Falcon Co.). Cells were routinely passed in 0.25% trypsin solution (Difco, 1:300) every 4 days. For the experiments, the cells at 4 days after subculture were dissociated with a solution of 0.02% disodium versenate (Inbar & Sachs, 1969*a*) and seeded in EM with 10% fetal calf serum. There was no detectable mycoplasma contamination as shown by testing the cultures on mycoplasma agar according to Chanock, Hayflick and Barile (1962). Total protein per cell was measured by the method of Lowry, Rosenbrough, Farr and Randall (1951). The volume of cells was measured after they were dissociated with a solution of 0.02% disodium versenate, by centrifuging a known number of cells in a centrifuge tube containing a graduated capillary tube (Inbar & Sachs, 1969*b*). The surface area was calculated from the volume assuming that the cells are spherical.

Concanavalin (Con. A)

Con. A was prepared from Jack bean meal (Sigma Chemical Co.) by two crystallizations (Sumner & Howell, 1936), and stored as a solution in saturated NaCl at room temperature. The protein was obtained from Miles Yeda. ^{63}Ni-labeled Con. A was prepared as described previously (Inbar & Sachs, 1969*b*) and stored as a solution at 4 °C. Batches of ^{63}Ni-labeled Con. A were stored for not more than 2 to 3 weeks. ^{63}Ni was obtained from the Radiochemical Center (Amersham, Eng.).

Cell Layers for Con. A Binding and for Transport Measurements

Binding of Con. A and the effect of Con. A on the transport of amino acids and carbohydrates in normal and transformed cells were studied on growing cell layers. Normal and transformed hamster cells were seeded at 7×10^5 cells per 50 mm Petri dish. The normal and transformed cells were cultured for 48 and 24 hr, respectively. Under these conditions, both types of cells underwent one cell generation and reached a density of about 15×10^5 cells per plate when they were used in the experiments. To test for binding of Con. A or the effect of the protein on transport, cell layers were treated with labeled or native Con. A at a concentration of up to 500 μg/ml for up to 120 min at 37 °C. The results indicate that this treatment did not affect the number of cells or the attachment of cells to the surface of the plate, as compared to the untreated controls. Treatment of cell layers with Con. A for more than 3 hr affected the attachment of the cells, which became rounded and detached from the plate. Binding of ^{63}Ni Con. A and transport measurements are expressed as counts/min/1,000 μg cell protein.

Assay for Con. A Binding to Cell Layers

For the binding assay with labeled Con. A, cell layers were washed twice with phosphate-buffered saline (PBS), pH 7.2, after removing the growth medium by aspiration. To test for binding, 1.5 ml of labeled protein diluted either in PBS or in a solution

of PBS containing 0.1 M α-methyl-D-glucopyranoside (α-MG) was added to the washed cell layers at a final concentration of 500 μg/ml (Inbar & Sachs, 1969*b*). The α-MG was added in excess to saturate the binding sites of Con. A. After incubation at 37 °C for 60 min, the cells were washed three times with 5 ml of PBS. The cells were then lysed with a solution of 0.1 N NaOH, aliquots were suspended in Bray's solution (Bray, 1960), and the radioactivity counted. To calculate the amount of labeled Con. A which is bound specifically (Inbar & Sachs, 1969*b*) by its α-MG binding sites, the amount bound in the presence of α-MG was subtracted from the amount bound in the absence of α-MG. The number of molecules bound was determined from the observation that 1 mg of the labeled Con. A used in these experiments gave 60,000 cpm, and the molecular weight of the protein was taken as 55,000 (Yariv, Kalb & Levitzki, 1968; So & Goldstein, 1968). In the present experiments, the binding of Con. A always refers to the α-MG specific binding. The α-MG specific binding was in all cases about 50% of the binding of Con. A in the absence of α-MG.

Assay for Amino Acid Transport

To study the effect of Con. A on the transport of amino acids, the following assay was used. Cell layers were washed twice with PBS followed by incubation with 1.5 ml Con. A diluted in PBS containing 0.1% glucose (Foster & Pardee, 1969) or with Con. A diluted in PBS-glucose containing 0.1 M α-MG. After periods of incubation at 37 °C, the Con. A solution was replaced with 1.5 ml of amino acid transport medium. The amino acid transport medium consisted of PBS containing 0.1% glucose and ^{14}C-labeled amino acid at a concentration of 2.0 mM and specific activity of 0.2 mC per mmole. After an uptake interval, the transport medium was rapidly removed by aspiration and followed by three gentle washes, each with 5 ml of iced PBS. Cells were then lysed with the NaOH solution, and the radioactivity counted in Bray's solution.

To calculate the effect of Con. A on transport, the amount of amino acid accumulated after treatment with Con. A in the absence of α-MG was subtrated from the amount accumulated after treatment in the presence of α-MG. The presence of 0.1% glucose either during Con. A treatment or in the transport medium did not interfere with the effect of Con. A on transport, and the presence of α-MG did not interfere with the transport of amino acids. Omitting glucose from the transport medium decreased the transport of all amino acids tested by about 25%. This indicates, as also found by Foster and Pardee (1969), that transport of amino acids under the present conditions was due to active transport rather than extracellular trapping of substrate. The routine assay for transport of amino acids was carried out as follows. Cells were treated with 500 μg Con. A per ml for 60 min followed by incubation with amino acid transport medium for 5 min at 37 °C. Under these conditions, about 5% of the total amino acid transport into the cell was accumulated into cell protein as measured by cold trichloracetic acid precipitation. Isotopically labeled amino acids were obtained from Calbiochem Inc. and from the Radiochemical Center (Amersham, Eng.).

Assay for Carbohydrate Transport

To study the effect of Con. A on carbohydrate transport, cell layers were washed twice with PBS followed by incubation with 1.5 ml Con. A diluted in PBS. Control cells were incubated with PBS only. After periods of incubation at 37 °C, the Con. A solution was replaced with 1.5 ml of carbohydrate transport medium. The carbohydrate transport medium consisted of PBS containing a ^{14}C-labeled carbohydrate at a concentration of 1.0 mM and specific activity of 0.5 mC per mmole. After an uptake interval, the radioactivity was measured as for the amino acid transport assay. Isotopically labeled carbohy-

drates were obtained from Calbiochem Inc., New England Nuclear, and Radiochemical Center (Amersham, Eng.).

To calculate the effect of Con. A on carbohydrate transport, the amount of substrate accumulated after treatment with Con. A was subtracted from the amount accumulated in untreated control cells. In contrast to the amino acid transport assay, the transport medium for carbohydrates as well as the binding of Con. A were carried out in the absence of glucose and α-MG. The transport assay for carbohydrates was routinely carried out as follows. Cells were incubated with 500 μg Con. A per ml for 60 min followed by incubation with a carbohydrate transport medium for 10 min at 37 °C.

Results

Binding of Con. A Molecules to Cell Layers

In order to determine the binding of Con. A to normal and transformed growing cell layers, the α-MG specific binding of ^{63}Ni-labeled Con. A was measured. The results indicate (Fig. 1) that, after 60-min incubation, the binding of Con. A molecules reached saturation. The saturation of Con. A binding sites was not due to an insufficient amount of Con. A, since the number of free Con. A molecules in the incubation mixture was about 20-fold higher than the number of Con. A molecules bound to the cells. The number of Con. A molecules bound per cell at saturation was calculated. The data

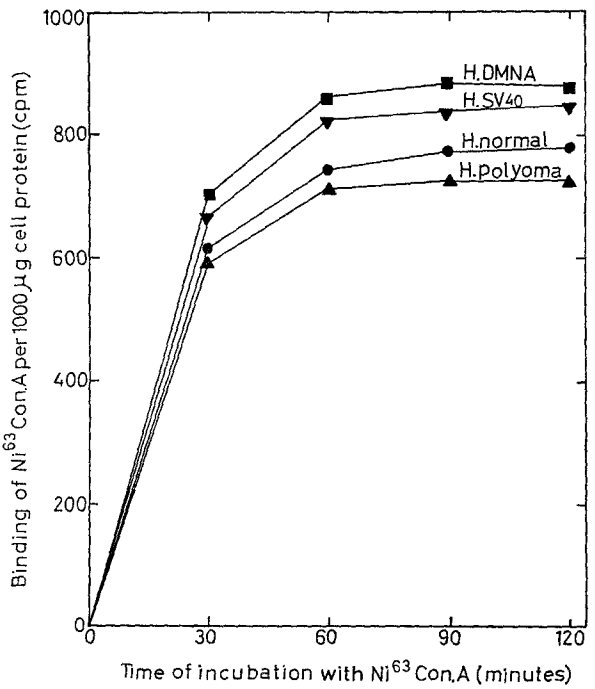

Fig. 1. Binding of ^{63}Ni Con. A (500 μg/ml) to normal and transformed hamster layers

Table 1. *Binding of Con. A to normal and transformed cell layers*[a]

Cells	No. of cells per plate ($\times 10^{-6}$)	Total protein per 10^6 cells (μg)	α-MG specific binding per 10^6 cells (cpm)	Surface area[b] per cell (μ^2)	No. of Con. A molecules bound per cell ($\times 10^6$)
H. Normal	1.45	318	229	1,106	38
H. Polyoma	1.50	316	223	1,079	37
H. DMNA	1.39	287	247	1,085	41
H. SV40	1.44	295	242	1,001	40

[a] Cells were incubated with 500 μg ^{63}Ni Con. A per ml for 60 min at 37 °C.

[b] Surface area per cell was claculated from the cell volumes after dissociating the cells with a solution of 0.02% disodium versenate (Inbar & Sachs, 1969*b*).

indicate (Table 1), that normal and polyoma-, SV 40- and DMNA-transformed hamster cells bound a similar number of Con. A molecules per cell. Since the total protein per cell and the cell volume after the cells were dissociated with a 0.02% disodium versenate solution were similar for normal and transformed cells, both types of cells bound a similar number of Con. A molecules per μ^2 cell surface.

Transport of Amino Acids

Experiments were undertaken to determine: (1) if binding of Con. A to the cell surface can result in an inhibition of amino acid transport and, if so, (2) if the difference in the structure of the surface membrane between normal and transformed cells can result in a differential inhibition of amino acid transport in the two types of cells.

Normal and polyoma-transformed hamster cells were treated with four concentrations of Con. A. The results indicate that Con. A inhibited the transport of L-leucine only in polyoma-transformed cells (Fig. 2, left). Maximum inhibition was obtained with a concentration of 500 μg per ml, after 60 min of incubation (Fig. 2, right). The transport of L-leucine into normal cells was unaffected under the same conditions.

The next experiments were carried out to determine the inhibitory effect of Con. A on the transport of three other metabolizable and two non-metabolizable amino acids in polyoma-, SV 40- and DMNA-transformed cells compared to normal hamster cells. Since maximum inhibition of L-leucine transport in polyoma-transformed cells was obtained after treatment with 500 μg Con. A per ml for 60 min, transport of the metabolizable amino acids (L-arginine, L-glutamic acid and L-glutamine) and of the non-

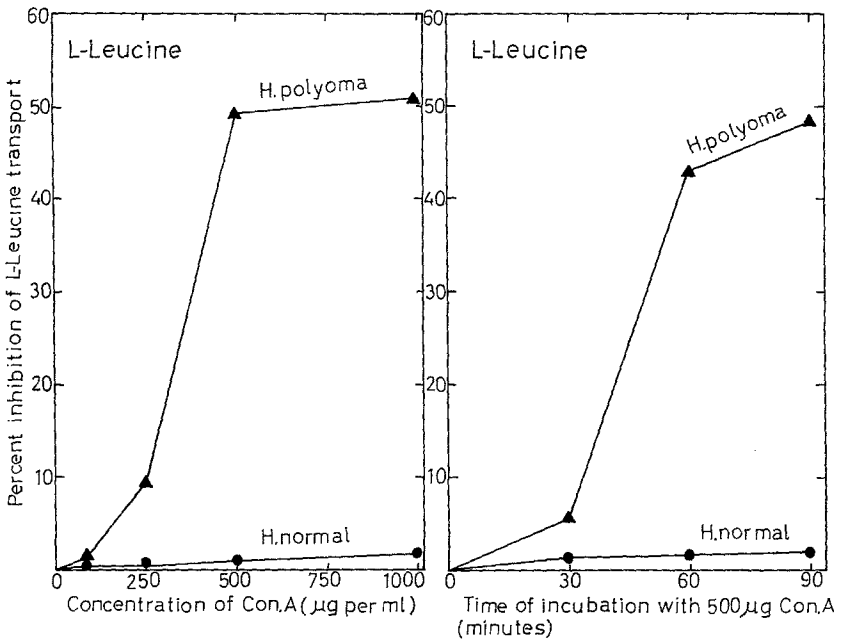

Fig. 2. Transport of L-leucine in normal and polyoma-transformed hamster cells. Transport after Con. A binding in the absence of α-MG is expressed as percent inhibition of the transport in the presence of α-MG. *Left*: cells incubated with Con. A for 60 min, followed by incubation with transport medium for 5 min. *Right*: cells incubated with 500 μg Con. A per ml up to 90 min, followed by incubation with transport medium for 5 min

metabolizable amino acids (α-aminoisobutyric acid and cycloleucine) was measured under these conditions. The results indicate (Table 2) that the binding of Con. A inhibited the transport of all metabolizable and non-metabolizable amino acids into transformed hamster cells, but had no effect on the transport of these amino acids into normal hamster cells. The kinetics of the effect of Con. A have also indicated (Fig. 3) that the rate of accumulation of the metabolizable amino acid L-leucine and the non-metabolizable amino acid cycloleucine was inhibited only in transformed cells and not in normal cells. These results indicate that the location of amino acid transport sites is different in normal and transformed cells in relation to the binding sites for Con. A.

Similar results were obtained with normal and polyoma-transformed rat cells. However, in the case of mouse cells, binding of Con. A gave a 20% inhibition of the transport of amino acids in normal SWR embryo cells compared to a 50% inhibition in SV 40-transformed SWR mouse cells.

When cells were treated with Con. A in the presence of α-MG, transformed hamster cells showed a more rapid transport of L-leucine and

Table 2. *Transport of amino acids after binding of Con. A to cell layers*[a]

Amino acids	Transport of amino acids after Con. A binding (cpm per 1,000 μg cell protein)											
	H. normal			H. polyoma			H. DMNA			H. SV40		
	+α-MG	−α-MG	%inhibition	+α-MG	−α-MG	%inhibition	+α-MG	−α-MG	%inhibition	+α-MG	−α-MG	%inhibition
L-leucine	5,390	5,297	2	9,407	4,913	48	8,303	5,341	36	8,547	5,060	41
L-arginine	3,115	3,018	3	3,216	1,471	54	3,377	1,727	49	3,466	1,913	45
L-glutamic acid	4,117	4,011	2	4,383	2,516	42	4,543	2,647	42	4,305	2,881	33
L-glutamine	15,332	14,580	5	15,470	7,033	45	15,733	8,145	47	15,896	6,918	56
Cycloleucine	16,722	15,871	5	19,443	8,213	58	17,870	8,245	54	18,765	8,230	56
α-aminoiso butyric acid	9,667	9,602	1	13,463	7,970	41	12,966	8,537	34	13,076	7,078	46

[a] Cells were treated with 500 μg Con. A per ml in the presence and absence of α-MG for 60 min followed by incubation with amino acid transport medium for 5 min. The transport in untreated cells was similar to that in cells treated with Con. A in the presence of α-MG.

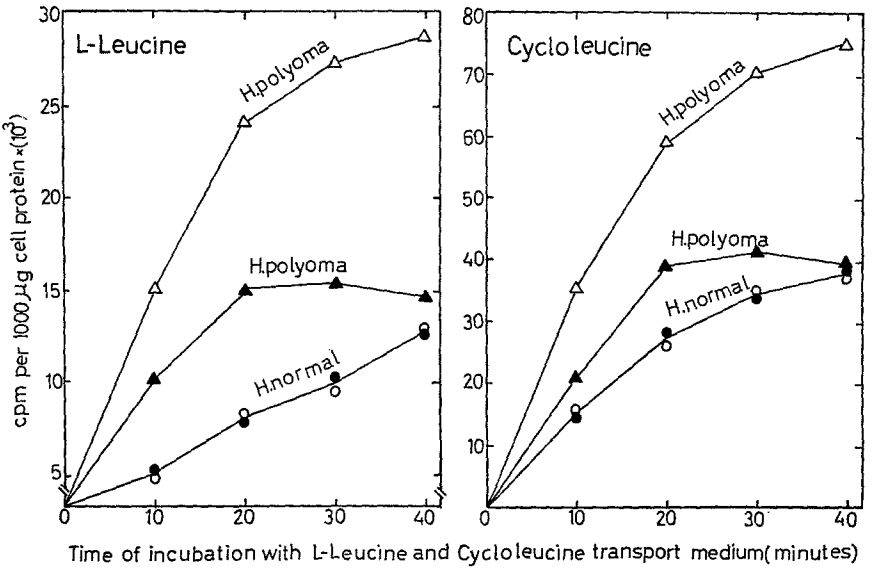

Fig. 3. Transport of L-leucine and cycloleucine in normal and polyoma-transformed hamster cells. Cells were incubated with 500 μg Con. A per ml for 30 min, followed by incubation with transport medium for up to 40 min. *Open symbols*: transport in the presence of α-MG. Closed symbols: transport in the absence of α-MG

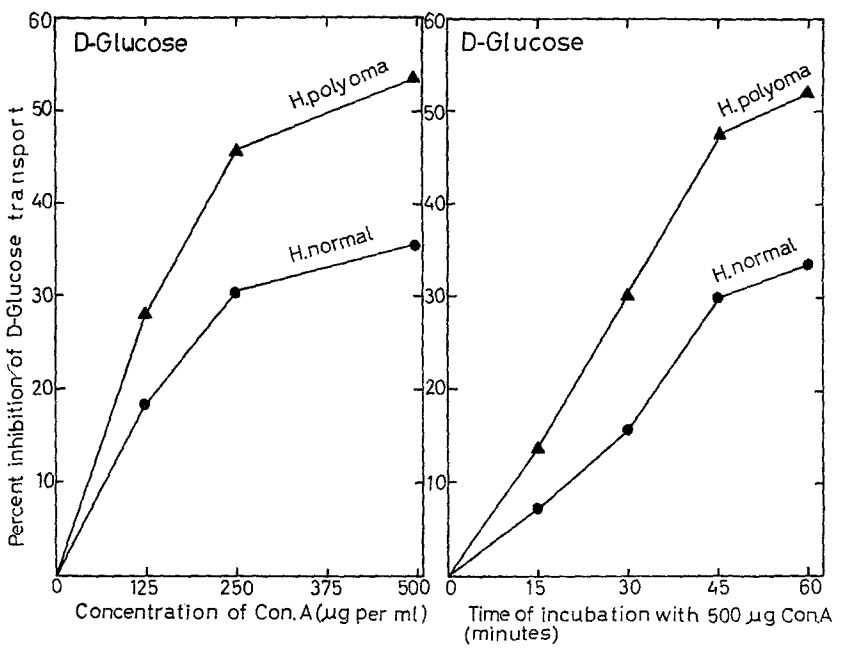

Fig. 4. Transport of D-glucose in normal and polyoma-transformed hamster cells. Transport after Con. A binding is expressed as percent inhibition of the transport in the absence of Con. A. *Left*: cells were incubated with Con. A for 60 min, followed by incubation with transport medium for 10 min. *Right*: cells were incubated with 500 μg Con. A per ml up to 60 min, followed by incubation with transport medium for 10 min

Table 3. *Transport of* D-*glucose and* D-*galactose after binding of Con. A to cell layers*[a]

Carbohydrate	Transport of carbohydrates after Con. A treatment (cpm per 1,000 μg cell protein)											
	H. normal			H. polyoma			H. DMNA			H. SV40		
	PBS	Con. A	%inhibition	PBS	Con. A	%inhibition	PBS	Con. A	%inhibition	PBS	Con. A	%inhibition
D-glucose	9,328	6,234	33	11,642	5,593	42	10,724	5,301	51	11,025	6,103	45
D-galactose	6,412	5,517	14	8,964	6,354	29	8,041	6,132	24	8,147	6,011	26

[a] Cells were treated with 500 μg Con. A per ml for 60 min, followed by incubation with carbohydrate transport medium for 10 min.

α-aminoisobutyric acid than normal hamster cells, although L-arginine, L-glutamic acid, L-glutamine, and cycloleucine were generally transported as rapidly by normal cells as by transformed cells (Table 2).

Transport of D-*Glucose and* D-*Galactose*

The effect of Con. A on the transport of D-glucose and D-galactose was also measured with normal and transformed hamster cells. The results indicate (Fig. 4, left) that Con. A inhibited the transport of D-glucose in normal and polyoma-transformed cells but that there was a higher inhibition in the transformed cells. Maximum inhibition for both types of cells was obtained with 500 μg Con. A per ml after 60 min (Fig. 4, right). A comparison of the effect of Con. A on the transport of D-glucose and D-galactose in normal and polyoma-, SV 40- and DMNA-transformed hamster cells has shown (Table 3) a greater inhibition for both carbohydrates in all the transformed cells tested. The results also indicate that inhibition by Con. A on the transport of D-glucose was higher than on D-galactose (Table 3). The

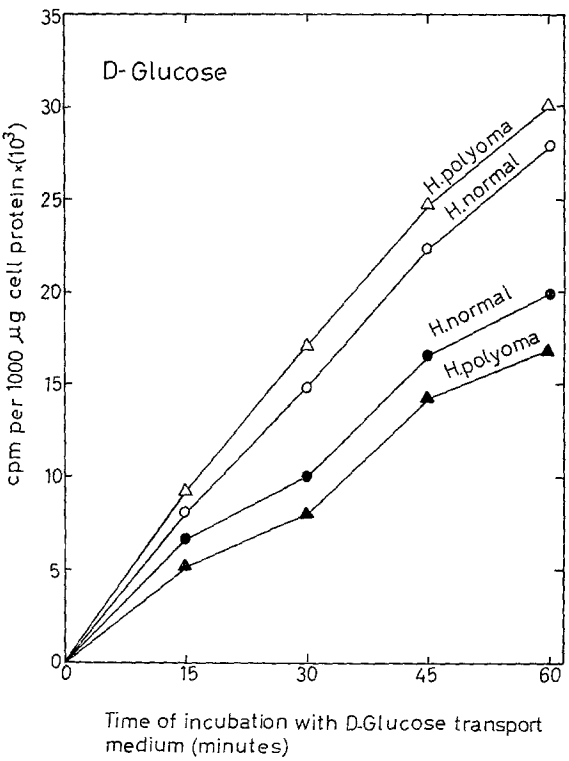

Fig. 5. Transport of D-glucose in normal and polyoma-transformed hamster cells. Cells were incubated with 500 μg Con. A per ml for 30 min, followed by incubation with transport medium up to 60 min. *Open symbols*: untreated control cells. *Closed symbols:* cells incubated with Con. A

kinetics of the effect of Con. A on the transport of D-glucose in normal and polyoma-transformed cells have indicated that the rate of accumulation of D-glucose (Fig. 5) was inhibited in both normal and transformed cells, and that there was a higher inhibition in the transformed cells. Untreated transformed cells transported D-glucose and D-galactose more rapidly than untreated normal cells. Similar results were obtained with normal and polyoma-transformed rat cells and with normal SWR mouse and SV 40-transformed SWR mouse cells.

Transport of L-*Fucose and Uptake of 3-0-Methyl-*D-*Glucose*

The rate of L-fucose transport (Fig. 6, left) was not affected by Con. A treatment in normal or polyoma-, DMNA- and SV 40-transformed hamster cells. This shows that the effect of Con. A on the transport of D-glucose and D-galactose and of amino acids was specific. There was also no effect of Con. A on the uptake of 3-0-methyl-D-glucose (Fig. 6, right). Since entry of 3-0-methyl-D-glucose into cells is a passive process (Weiss & Narahara, 1969), it can be assumed that binding of Con. A did not affect the permeability of the cell surface membrane as far as entry of 3-0-methyl-D-glucose is concerned. Similar results were obtained with normal and transformed rat and mouse cells.

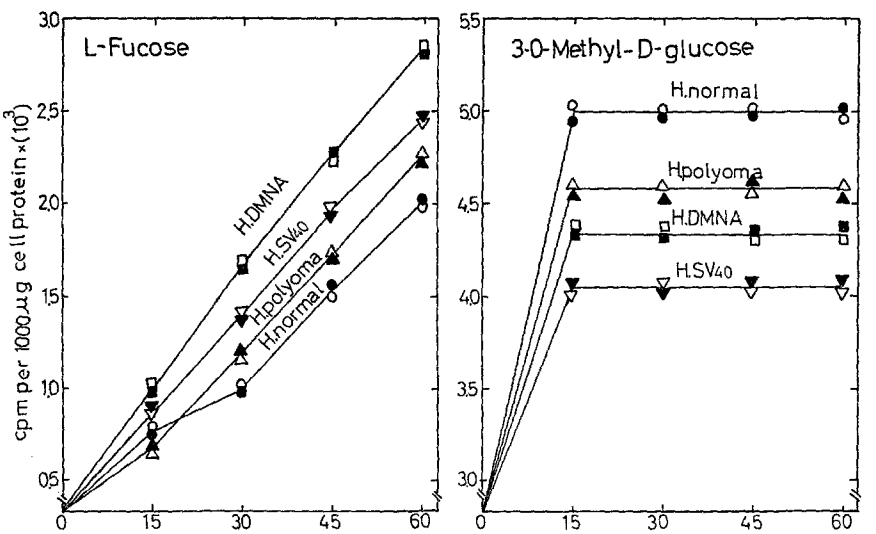

Fig. 6. Transport of L-fucose and entry of 3-0-methyl-D-glucose in normal and transformed hamster cells. Cells were incubated with 500 µg Con. A per ml for 30 min, followed by incubation with transport medium up to 60 min. *Open symbols:* untreated control cells. *Closed symbols:* cells incubated with Con. A

Discussion

The present results have indicated a specific differential effect of Con. A on the transport of amino acids and carbohydrates in normal and transformed cells. Experimental conditions were used so that a similar number of Con. A molecules were bound to both types of cells. The results have shown, that the binding of Con. A to the α-MG-like sites on the surface membrane can inhibit the active transport of four metabolizable and two non-metabolizable amino acids in transformed hamster cells, whereas under the same conditions normal hamster cells were not affected. The transport of D-glucose and D-galactose was inhibited by Con. A in both normal and transformed cells, but the inhibition was higher in transformed than in normal cells. In both types of cells, D-glucose was inhibited more than D-galactose. The effect of Con. A on transport was specific, since there was no effect on transport of L-fucose in all the cells tested. There was also no effect of Con. A on the uptake of 3-0-methyl-D-glucose, which is a passive process (Weiss & Narahara, 1969). It can be concluded that the binding of Con. A to the surface membrane has a direct effect on amino acid and carbohydrate transport sites, although the possibility that there is also an indirect effect cannot be excluded.

The present results can be used to locate amino acid and carbohydrate transport sites in relation to the binding sites for Con. A on normal and transformed cells. The results indicate that in normal cells Con. A sites are associated with transport sites for D-glucose and to a lesser extent D-galactose. In transformed cells, the Con. A sites are associated with transport sites for amino acids, and also with sites for D-glucose and D-galactose to a greater extent than in normal cells (Fig. 7). The malignant transformation of normal cells is thus associated with a change in the location of amino acid and carbohydrate transport sites in relation to the binding sites for Con. A.

Malignant transformation of normal cells, as far as the α-MG-like binding sites of Con. A are concerned, can be explained by three types of changes in the structural organizations of sites in the surface membrane. There can be an exposure of cryptic sites, a concentration of exposed sites by a decrease in cell size, and a rearrangement of exposed sites without a decrease in cell size (Ben-Bassat *et al.*, 1971). Rearrangement of the surface membrane in cell transformation is illustrated by the change in the location of amino acid and carbohydrate transport sites.

The difference in the structure of the surface membrane between normal and transformed cells, which includes a difference in metabolic activity (Inbar, Ben-Bassat & Sachs, 1971), can be reflected by a differential inhibition

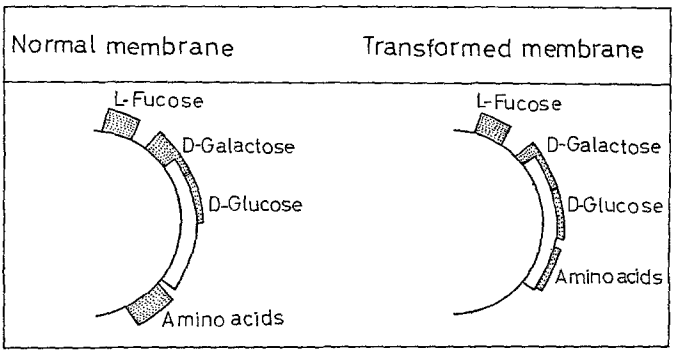

Fig. 7. Model of the location of amino acid and carbohydrate transport sites in relation to binding sites for Con. A, in the surface membrane of normal and transformed cells. □, Con. A binding sites

of cell multiplication and cell killing on normal and transformed cells *in vitro*, and local treatment of transplanted tumors with Con. A resulted in an inhibition of tumor development *in vivo* (Shoham *et al.*, 1970). It will be of interest to determine if this differential effect on normal and transformed cells is due to the differential effect of Con. A on the transport of amino acids and carbohydrates. It has been shown that the formation of cell variants with a reversion of properties characteristic of transformation, which have a different chromosome constitution than transformed cells (Rabinowitz & Sachs, 1970; Hitotsumachi, Rabinowitz & Sachs, 1971), is associated with a reversion of the structure of the cell surface membrane (Inbar, Rabinowitz & Sachs, 1969; Rabinowitz, Sela & Sachs, 1971). It will therefore also be of interest to determine the location of amino acid and carbohydrate transport sites in these reverted cells.

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