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INFLUENCE OF TEMPERATURE ON THE INTERACTION OF CONCANAVALIN A WITH CHICK FIBROBLASTS DURING EMBRYO DEVELOPMENT.

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The interaction between Concanavalin A and chick embryo fibroblasts was studied. Cells from younger and older embryos had the same number of lectin receptor sites per cell at 4°, 21° and 37°C but the affinity constants increased with increasing temperature. Analysis of the binding data according to Scatchard showed that the apparent changes in binding as a function of temperature might be related to thermodynamic properties. The lectin binding sites on the cell surface proved homogeneous with regard to binding properties.

The age-related differences noted in the affinities of the cells to bind Concanavalin A could be related to differences in the degree of rearrangement of the cell surface components and/or to a change in the structure of cell surface glycoconjugates, and may serve to explain the differences in the effect of Concanavalin A on cell growth.

INTRODUCTION

The mitogenic lectin, Concanavalin A (Con A),is known to induce marked agglutination in the cells of young embryos (1) and Kleinschuster and Moscona (2) have shown that there is a relationship between the rate of agglutination of embryo cells and the stage of development of the embryo. In addition, the effect of Con A on the proliferation of embryo cells differs with the stage of development (3-7), proliferation being stimulated in older cells and depressed in younger cells. It is well established that the effect of Con A involves an interaction of the lectin with the binding sites located on the cell surface and that this binding constitutes the first and a necessary step in the process (4). The differential effect of Con A on embryo cells could therefore be related to the number of lectin binding sites available. In the case of normal and transformed cells it has been shown (8-14) that the number of Con A receptor sites does not differ with differentiation. This may however be explained by the differences in

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experimental conditions since lectin binding is generally studied at 4°C, agglutination at 21°C and cell growth at 37°C. Noonan and Burger (15) have shown that the number of Con A binding sites remains unchanged at 4°C whereas marked differences appear at higher temperatures (22 and 37°C). The possibility of such variations existing in the number of Con A receptors has not yet been investigated in embryo cells as related to their stages of development. We have therefore studied the effects of temperature on Con A binding to chick fibroblasts at two stages of embryo development.

MATERIALS AND METHODS.

Cells.

Fibroblasts were obtained from 8- and 16- days chick embryos (Société Jourdain, France) by the method of Rein and Rubin (16), modified as previously described (3).

Cell cultures.

Primary monolayer cultures were made in 16mm diameter wells in 0.5 ml of Eagle's minimum (Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 1% glutamine, 1% antibiotics (penicillin, streptomycin) and 10% foetal calf serum. The initial seeding concentration was 10^6 cells/ml (0.5×10^6 cells/well). Cultures were grown in humidified air containing 5% CO_2 at 37°C. A sample of cells was counted in a haemocytometer. Each measurement refers only to viable cells and represents the mean of six samples. The experiments were done on subconfluent cultures (1×10^6 cells/well). Subconfluency in fibroblasts from 8-day embryos occurs at 48h. and in those from 16-day embryos at 96h., which reflects the differences in proliferative capacity of the cells at different stages of embryo development (5).

Concanavalin A

Concanavalin A (Con A), Grade III, was purchased from Sigma (St Louis, USA).

Labelling of Con A.

The lectin was labelled by the method of Miller and Great (17) using ^{14}C-acetic anhydride (specific activity 10-30 mCi/mmol ; The Radiochemical Centre, Amersham). Labelled lactin was purified by gel filtration on a Bio-Gel P6 column (2 x 40 cm), the eluting buffer being 0.005 M-$NaCO_2$ / 0.15 M-NaCl, pH 7 (Buffer A). The specific radioactivity of the lectin was 2×10^6 dpm/mg. The labelled lectin exhibited an erythroagglutination activity and a molecular weight (110 000) identical to those of unlabelled Con A. Indeed, labelled and unlabelled Con A were eluted with the same volume of elution using a Bio-Gel P200 column chromatography in phosphate buffer saline pH 7.4.

The labelled lectin solution was used at a final concentration of 1mg/ml in Buffer A.

Binding assay of (^{14}C)-Con A.

The binding assays were carried out at 4°, 21° and 37°C. They were performed with cell monolayers of chick embryo fibroblast cultures at sub-

confluency, as recommended by Noonan and Burger (18). Subconfluent monolayer cultures ($1.0 \pm 0.1 \times 10^6$ cells/well) were washed three times with phosphate buffer pH 7.4 and covered with a layer of 0.5 ml of phosphate buffer containing (^{14}C)-labelled Con A at concentrations varying from 1 to 80 µg/ml. The cells were incubated for 30min. and then washed three times with phosphate buffer pH 7.4 and dissolved in 0.5 ml of 0.1 N NaOH before being added to 10 ml of scintillation fluid (ACS, Amersham/Searle). To measure non-specific binding of (^{14}C)-labelled Con A, cultures were incubated with the labelled lectin in the presence of 0.1 Mαmethyl-D-mannopyranoside, a competitive inhibitor of Con A (19). Non-specific binding was subtracted from the total bound radioactivity in order to determine the specific binding of Con A. The radioactivity of each sample was counted in a liquid scintillation spectrometer (Intertechnique SL 30). Experimental data were plotted by the method of Scatchard (20) taking into account the molecular weight of the lectin (110 000). A Ti 58 calculator (Texas Instrument) was used to calculate the least square-fit to Scatchard plots of the data and to determine the values of Ka and the number of lectin binding sites. The correlation coefficient was 0.98-0.99. Each point is the average of triplicate determinations.

Dissociation of (^{14}C)-labelled Con A - cell complex.

The dissociation of (^{14}C)-Con A from the embryo cells was done according to the method of De Meyts et al (21). Monolayer cultures of chick embryo fibroblasts were preincubated with 1µg of (^{14}C)-Con A in 1ml of phosphate buffer pH 7.4 (10^6 cells/ml) for 30min. at 37°C. After the preincubation period the cells were washed three times with phosphate buffer pH 7.4. At zero time, the monolayer cultures were transfered to a 1000-fold higher volume (1000 ml) of phosphate buffer in the absence and the presence of unlabelled Con A (1000 µg/ml). At the indicated times, the amount of radioactivity remaining associated with the cells was measured as described above for the Con A binding experiments.

Thermodynamics of Con A - to - cell binding site interactions.

The thermodynamic functions for the binding process can be evaluated from the value of the apparent association constant K (22). The thermodynamic values of standard free energy change ($\Delta G°$) were calculated from K by the equation :

$$\Delta G° = - RT \ln K \qquad [1]$$

The effect of temperature on the binding process was evaluated from the enthalpy change ($\Delta H°$) using the equation :

$$\ln \frac{K2}{K1} = \frac{\Delta H°}{R} \times \left(\frac{1}{T2} - \frac{1}{T1} \right) \qquad [2]$$

where K1 and K2 are the apparent association constants at temperatures T1 and T2.

The standard entropy change ($\Delta S°$) was calculated from the equation :

$$\Delta G° = \Delta H° - T \Delta S° \qquad [3]$$

RESULTS AND DISCUSSION

I - Binding of ^{14}C-labelled Con A to the cells.

The fibroblasts rapidly bound Con A to plateau levels within 30min. at 4°, 21° and 37°C. Subconfluent cultures of the fibroblasts were saturated with the lectin at a concentration of 40 µg/ml.

For fibroblasts from 8- day embryos the Scatchard (20) plots of the specific binding of Con A at 30min. gave monophasic curves at all the temperatures

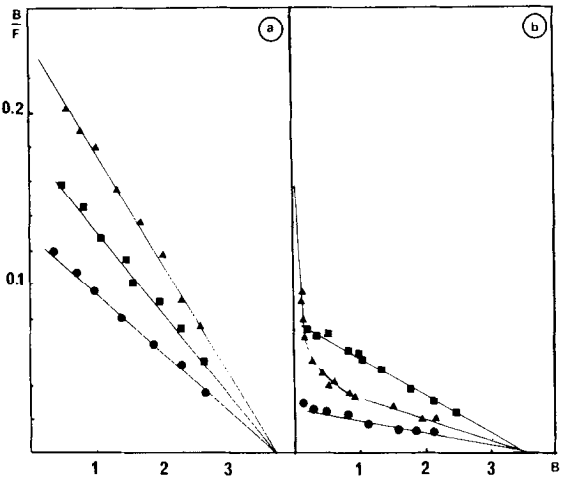

Figure 1 : Scatchard plots of the specific binding of ^{14}C-labelled Con A to fibroblasts from (a) 8-day, and (b) 16-day embryos at various temperatures, (●), 4°C, (■) 21°C and (▲) 37°C.
The cells were incubated for 30min. with Con A at concentrations varying from 1 to 80 µg/ml. B represents the amount of bound lectin and F the amount of free lectin (both in µg/10^6 cells). Experimental procedures are described in Materials and Methods. In our experiments, α methylmannopyranoside inhibited the lectin binding to cell monolayers by 80-90% at 4°C and 21°C and by 60% at 37°C in fibroblasts from both 8- and 16-day embryos.

studied, suggesting only one class of binding sites (Fig.1). Fibroblasts from 16- day embryos also exhibited a monophasic curve at 4°C and 21°C, but at 37°C a concave curve was noted, suggesting either heterogeneity of the lectin binding sites or negative co-operativity. The second possibility seemed to fit our results better since i) at 4°C and 21°C the line of the Scatchard plots had a single slope, which would suggest a single class of binding site, this suggested that at 37°C the binding sites in the 16- day cells were homogenous, ii) in addition, at 37°C the apparent affinity constant decreased with binding site occupancy. In order to assess the decrease in Ka, we used the direct kinetic method of De Meyts and Roth (23), who consider affinity as a variable or average property, and related it directly to the occupancy of the binding sites so as to demonstrate the site-site interactions of the negative co-operative process involved in the Con A binding to the 16- day fibroblasts at 37°C. When all the sites were empty the Ka was 5.1 x 10^6 M^{-1} and when all the sites were occupied the Ka was 1.5 x 10^6 M^{-1}, iii) finally the conclusion that the Con A-binding to the 16- day fibroblasts at 37°C was related to a negative co-operative process is further supported by the observation shown in Fig.2 that the spontaneous rates of dissociation of the labelled Con A is accelerated in the presence of a large excess of unlabelled Con A.

In contrast to the affinity constant which depends on temperature and on the age of the embryos (Table I), the number of Con A binding sites

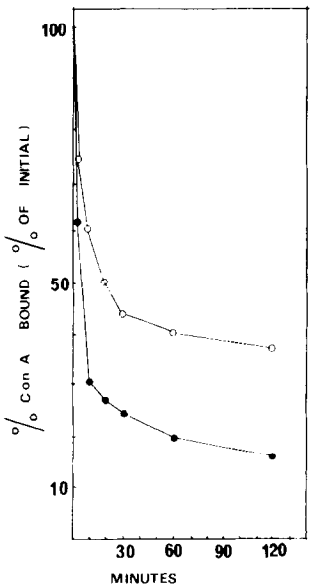

Figure 2 : Dissociation of the ^{14}C-labelled Con A bound to fibroblasts from 16-day embryos as a function of time at 37°C in the absence (○) and the presence of a large excess of unlabelled Con A (●). The monolayer cultures are processed as described in Materials and Methods. Each point is the mean of values obtained from two separate experiments done in triplicate and standard error does not exceed 10%.

($1.9 - 2 \times 10^7$ /cell) was related to neither (Table I). These results are at variance with those reported by Noonan and Burger (15) and Huet et al (24) who used normal and virus-transformed cell lines, but are very similar to both those reported by Birdwell and Strauss (25) for normal chick embryo fibroblasts in secondary culture (1.5×10^7 /cell) and those for mam-

TABLE I

Age of embryos (days)	Temperature (°C)	Binding sites (number/cell)	Affinity constant kA M^{-1}
8	37	2×10^7	7.4×10^6
	21	2×10^7	5.6×10^6
	4	2×10^7	4×10^6
16	37	1.9×10^7	$k\bar{A}_f$, 1.5×10^6
			$k\bar{A}_e$ 5.1×10^6
	21	1.9×10^7	2.4×10^6
	4	1.9×10^7	0.84×10^6

Quantitative interactions between Con A and chick embryo fibroblasts at various stages of development. Experimental procedures are described under Materials and Methods. The number of Con A binding sites and the affinity constants were obtained by the method of Scatchard (20) and the range of Con A concentrations used was 1-80 µg/ml. Each result gives the range of values obtained from four separate experiments.

$k\bar{A}_f$: apparent affinity constant when all sites were filled : $k\bar{A}_e$: apparent affinity constant when all sites were empty. These were determined by graphic analysis of the De Meyts and Roth (22) plots.

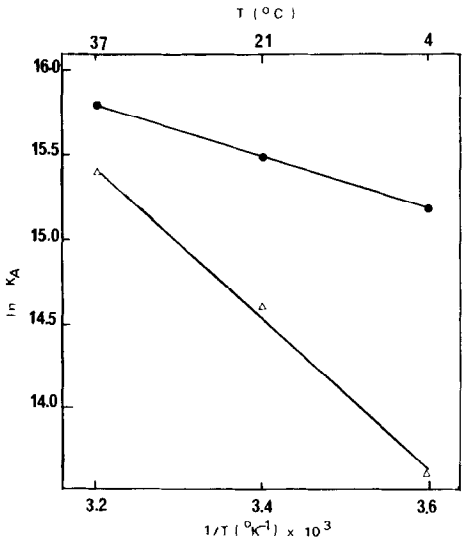

Figure 3 : Van't Hoff plot of the Con A binding to fibroblasts from (●) 8-day and (△) 16-day embryos. The affinity constant Ka was determined at 4, 21 and 37°C from Scatchard plots and plotted as a function of the reciprocal of the absolute temperature.

malian cell lines such as normal and transformed 3T3 and BHK cells (8-10, 26). The question which now arises is how to explain the differences in affinity constant related to temperature and age noted in the fibroblasts from 8- and 16- day embryos. The change in affinity constant could be attributable to a redistribution of the Con a binding sites within the membrane as facilitated by the fluid character of the membrane (26-27). Local modifications of membrane fluidity have been observed in chick embryo hearts during their development (28) and human erythrocytes during in vivo aging (29). At the molecular level, the fluidity of the cell membrane is governed by the composition of the lipid double layer, i.e., the ratio between cholesterol and phospholipids and the amount of unsaturated fatty acid (28-31). We have previously found that fibroblasts from younger embryos contain more unsaturated fatty acids and more short-chain lipid molecules (32) showing that cell membrane fluidity decreased within embryo development, which would suggest that there might be an increased in entropy in the membrane during the binding process owing to the release of low-entropy water previously trapped electrostatically in the double layer (33-37).

II - Thermodynamics of the Con A - to - cell binding site interaction.

The thermodynamic data for the binding of Con A are given in Table II. The apparent affinity constant of the association between Con A and cell surface binding sites varied markedly with temperature (Table I).
The Van't Hoff plots of the data (Fig.3) were linear for the three tempe-

TABLE II

Age of embryos (days)	Temperature (°C)	$-\Delta G^\circ$ (kcal/ml.)	ΔH° (kcal/mol.)	ΔS° (cal.deg^{-1} mol^{-1})
8	4	8.4	3.1	41.53
	21	9.1		
	37	9.8		
16	4	7.5	7.8	55.13
	21	8.6		
	37	9.5		

Thermodynamic functions of the interactions between Con A and the cell binding sites in fibroblasts from chick embryos at various stages of development.
These functions were calculated as described in Materials and Methods taking into account the values of the apparent affinity constants given in Table I.
Each result gives the range of values obtained from four separate experiments.

ratures studied for fibroblasts from both 8- and 16- day embryos. The Con A-specific binding process therefore exhibited a normal behaviour at all the temperatures studied, indicating that there was neither a large change in heat capacity nor any major change in the proteins over the temperature range studied, which might affect the binding mechanism.

Enthalpy changes were determined using equation 2. These data showed that the interactions between Con A and the cell surfaces had a positive reaction enthalpy, whereas those between Con A and small saccharides had a negative reaction enthalpy (35). Our results are similar to those reported for Con A and various other cell types (35-40) but very different from those reported for wheat germ agglutinin (41-43) where Ka decreases slightly when the temperature is raised, yielding a negative reaction enthalpy similar to that measured for the formation of antibody haptens (40). As is shown in Table II, the changes in both enthalpy and entropy for the association of Con A with the binding sites increased with the age of the embryos. This suggests that the reaction was driven by entropy, enthalpy working against it. The increase in positive entropy observed in the Con A binding to the fibroblasts between the 8th and the 16th day of development agrees with the notion mentioned above concerning the release of low-entropy water (33-41). The larger standard entropy change and the co-operativity of the interaction between Con A and cells suggest that the fibroblasts from 16- day embryos the Con A binding process might result in a greater degree of re-arrangement of the cell surface components than it does in those from 8- day embryos. The less extensive re-arrangement would coincide with the lack of co-operativity in the younger cells.

Thus age-related differences noted in the affinity constants of the Con A binding with fibroblasts from 8- and 16- day embryos, according

to the experimental temperature could be related to differences in the degree of re-arrangement of the cell surface components and/or to a change in the structure of cell surface glycoconjugates and may serve to explain the differential effects of Con A on cell growth.

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