

## SPONTANEOUS AND LECTIN-INDUCED REDISTRIBUTION OF CELL SURFACE RECEPTORS ON EMBRYONIC CHICK NEURAL RETINA CELLS

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### SUMMARY

The mobility of plant lectin receptors in the plane of the membrane is examined for cells prepared from embryonic chick neural retinas by a variety of procedures. Cells liberated from the intact tissue by trypsin treatment followed by mechanical dissociation are able to redistribute their receptors into 'caps' both spontaneously and in the presence of a multivalent lectin. These cells, dispersed by trypsinization, upon repair in culture for a suitable period of time lose their ability to redistribute lectin receptors. Cells dispersed by mechanical means without prior trypsin treatment are unable to undergo 'cap' formation. In addition, cells within intact tissues are also unable to redistribute their lectin receptors into 'caps.' Based on these observations we propose that within solid tissues which have assumed their characteristic architecture, cell surfaces are immobilized, and that this phenomenon may be a critical parameter in determining the potential of a cell to undergo morphogenetic rearrangements.

### INTRODUCTION

Studies on the mechanisms of cell recognition and adhesion most often rely on the use of single cell suspensions prepared by mild trypsin treatment and subsequent mechanical dissociation. The effects of this treatment on membrane structure in general, and on those components involved in recognition and adhesion in particular, are unknown. It is of critical importance in understanding the mechanisms which mediate cell adhesion to understand how the cell surface is altered during single cell preparation.

Two effects of trypsin treatment which may have implications for studies on cell-cell interactions have been reported: (1) agglutination of untransformed cells is greatly facilitated by trypsinization and approaches the level of transformed cells (Burger, 1969); and (2) lateral mobility of lectin receptors in the plane of the membrane increases following trypsinization, resulting in altered surface receptor distributions (Nicolson, 1972; Inbar, Huet, Oseroff, Ben-Bassat & Sachs, 1973). Originally, it was proposed that trypsin treatment merely exposed cryptic lectin-binding sites (Burger, 1969); but more recently, to account for both increased agglutinability and altered lectin receptor distribution, Nicolson (1972) has suggested that trypsin causes a spontaneous reorganization of membrane components.

While it appears that mobility of cell surface receptors in the plane of the membrane is a property common to many types of single cells, it is not yet clear whether

this is also true for cells within tissues or if any relationship exists between receptor mobility and the processes of tissue formation. In addition, it is not clear whether reformation of cell-cell contacts and histogenesis in reaggregating suspensions of embryonic cells is dependent upon a particular distribution of surface components. Embryonic chick neural retina has been used extensively for studies on the mechanisms of adhesion and *in vitro* histogenesis and as such offers a possible means of examining these relationships. We report here on the mobility of lectin receptors on cells prepared by 3 different means: trypsinization of whole tissues with subsequent mechanical dissociation, mechanical dissociation of whole tissues after removal of divalent cations, and mounting of intact tissue fragments without dispersal into single cells.

#### MATERIALS AND METHODS

##### *Preparation of single cells*

Neural retinas were obtained from 10-day White Leghorn chick embryos by dissection in warm Tyrode's solution gassed to pH 6.5 with CO<sub>2</sub>. Three retinas were placed in a 10-ml Erlenmeyer flask and dissociated by one of the following procedures:

**Trypsinization.** Tissues were washed once with warm pH 7.2 Hepes (0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulphonic acid) buffered saline with glucose (1 mg/ml, HBSG) and then incubated for 15 min at 37 °C with gentle rotation (40 rev/min) in 3 ml of HBSG (pH 7.4) containing 4000 units/ml of trypsin (Miles, 3 × crystalline). The tissues were then washed 3 times with warm Tyrode's solution (pH 6.5) and finally dissociated in 3 ml of Tyrode's solution (pH 6.5) containing 50 µg/ml of DNase (NBC, crystalline) by flushing through a Pasteur pipette with the tip flamed to an opening of 0.5 mm. The dissociated cell suspension was immediately cooled in an ice waterbath.

**Mechanical dissociation.** Three whole neural retinas were incubated at 37 °C in three 10-min changes of 3 ml of either calcium- and magnesium-free Tyrode's solution (CMF) or 0.1% ethylene diamine tetraacetic acid (EDTA) in HBSG. After washing 3 times with Tyrode's solution (pH 6.5) the tissues were dissociated as above. The cell suspension was then filtered through 5 layers of lens paper to eliminate large cell clusters.

##### *Redistribution assays*

Fluorescein isothiocyanate-conjugated concanavalin A (FITC-Con A, Miles) and fluorescein isothiocyanate-conjugated wheat germ agglutinin (FITC-WGA, Miles) were used at concentrations of 40–60 µg/ml in HBSG for a suspension containing 20 × 10<sup>6</sup> cells/ml. Binding was carried out for 20 min at 0 °C. In all cases, binding was abolished by co-treatment with 0.2 M  $\alpha$ -methyl-D-mannoside (for FITC-Con A) or 0.2 M *N*-acetyl-D-glucosamine (for FITC-WGA).

In order to determine the initial distribution of lectin receptors, 20 × 10<sup>6</sup> freshly dispersed cells were spun at 200 g for 5 min at 4 °C, resuspended in 1 ml of 2% glutaraldehyde (Ladd) in 0.05 M sodium acetate buffer (pH 4.0) or 2% glutaraldehyde in 0.2 M NaCl in sodium phosphate buffer (0.01 M, pH 7.2), and fixed for 30 min at 4 °C. Both of these fixation procedures prevented any change in surface receptor distribution. After washing the cell suspension 3 times with HBSG, the cells were labelled with lectin. Following incubation in lectin solution, the cells were again washed with HBSG and examined under a Zeiss Universal microscope with an epifluorescence system and 40 × oil-immersion objective. Randomly chosen fields of 100 cells were scored for ring, patch and cap configurations of fluorescent labelling (Fig. 8).

Lectin-induced redistribution of receptors was followed by washing 20 × 10<sup>6</sup> freshly dissociated cells twice with 3 ml of HBSG at 4 °C, resuspending in 1 ml of lectin solution, incubating for 20 min at 4 °C, washing twice in the cold with 1 ml of HBSG and incubating in 1 ml of HBSG for 10, 30, or 60 min at 22 °C. The cells were then fixed as described above and resuspended in HBSG after washing for observation. Spontaneous redistribution of receptors was

followed in the same way with the binding of lectin for 20 min at 4 °C following incubation at 22 °C and fixation at 4 °C.

The effects of various inhibitors on lectin receptor redistribution were assessed by adding the inhibitor at the onset of incubation at 22 °C (spontaneous condition) or during binding of lectin as well as during incubation at 22 °C (lectin-induced condition). Cytochalasins B and E (Aldrich Chemical Co.) were dissolved in dimethyl sulphoxide (DMSO). Colchicine was purchased from Sigma. Lumicolchicine and griseofulvin were gifts from Dr Gary Borisy.

### Agglutination assay

Five million unfixed or fixed cells in 0.2 ml were added to 0.1 ml of Con A at various concentrations in wells of a Linbro multiwell dish. After incubation for 30 min at 22 °C on a rotating platform (80 rev/min) the wells were examined for agglutination. The lowest dilution of lectin at which any observable agglutination occurred was used as a measure of agglutinability.

## RESULTS

### Redistribution of surface receptors on single cells prepared by trypsinization

When the distribution of Con A receptors was followed over time at 22 °C using cells prepared by trypsin treatment and which had not been exposed to lectin prior to fixation (spontaneous condition, see Materials and methods) it was observed that the proportion of cells showing ring fluorescence decreased rapidly during the first 10 min and then more slowly to a level of 37% after 60 min. Concurrently, the

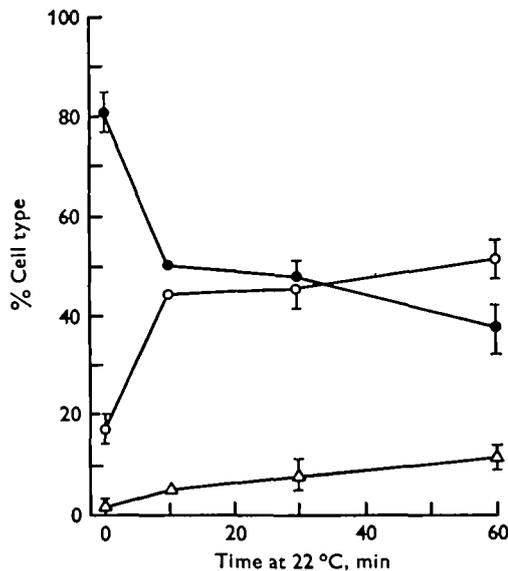


Fig. 1

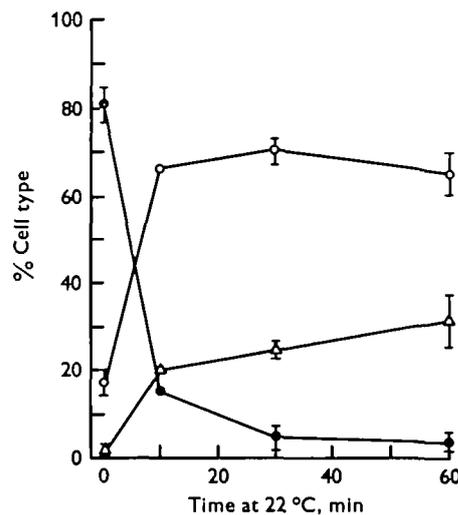


Fig. 2

Fig. 1. Spontaneous redistribution of Con A receptors during incubation at 22 °C. Symbols represent the percentage of the population of cells having ring (●), patch (○), or cap (Δ) fluorescence. In all figures, error bars represent the standard error of the mean from duplicates in 3-5 separate experiments. For clarity, in some cases only half of the error bar is presented.

Fig. 2. Lectin-induced redistribution of Con A receptors during incubation at 22 °C. Symbols as in Fig. 1.

percentage of cells having a patch distribution of receptors increased rapidly during the first 10 min and less rapidly thereafter, approaching a level of 51% after 60 min. The percentage of cells with caps showed a continuous slow rise to 12% throughout the 60-min incubation (Fig. 1).

A similar time course of redistribution was observed with trypsin-prepared cells after pre-incubation in lectin solution (lectin-induced conditions, see Materials and methods). However, redistribution was more extensive under lectin-induced conditions. The lectin-induced redistribution of Con A receptors was also characterized by a rapid decrease in rings and a simultaneous increase in patches during the first

Table 1. *Inhibition of capping of Con A receptors by high concentrations of Con A*

Con A concentration, $\mu\text{g/ml}$	Degree of inhibition of patching, %	Degree of inhibition of capping, %
50	0	5
100	0	33
200	0	63
400	0	74

FITC-Con A at various concentrations was added to  $20 \times 10^6$  cells in HBSG which were assayed for the percentage of the population of cells having caps after 60 min as described in Materials and methods.

10 min at 22 °C followed by a slower rate of change thereafter, approaching a level of 70% by 60 min. The percentage of cells with caps showed a continuous increase throughout the incubation period, reaching 31% at 60 min (Fig. 2). Concentrations of Con A greater than 75  $\mu\text{g/ml}$  significantly inhibited capping of receptors on trypsin-dispersed cells (Table 1) while patching was unaffected.

The effect of blocking the mobility of one receptor on the redistribution of a second was studied by adding unlabelled Con A at a concentration of 200  $\mu\text{g/ml}$  to cells labelled with FITC-WGA in the redistribution assay. High concentrations of Con A had no effect on the redistribution of WGA receptors. Similarly, unlabelled WGA (200  $\mu\text{g/ml}$ ) had no effect on the redistribution of Con A receptors. The receptors for these 2 lectins appear to redistribute independently of each other in the plane of the membrane.

To characterize further the phenomena of patch and cap formation of Con A receptors on trypsin-dispersed retina cells the effects of various inhibitors on both spontaneous and lectin-induced redistributions were examined. Sodium azide (1 mM) had no effect on patch formation but completely inhibited capping under both spontaneous and induced conditions. Cycloheximide (5  $\mu\text{g/ml}$ ) had no effect on patching or capping under either condition.

Since both microtubules and microfilaments have been implicated in the process of lectin receptor redistribution, several drugs whose biological effects are thought to be related to disruption of microtubules and microfilaments were tested. Colchicine had a concentration-dependent effect, inhibiting capping by approximately 60% at a

concentration of  $40 \mu\text{g/ml}$  in both the lectin-induced and spontaneous cases (Figs. 3, 4). Lumicolchicine, an ultraviolet irradiation product of colchicine which does not disrupt microtubules (Wilson & Friedkin, 1967), had no effect. In addition, griseofulvin, a drug possessing similar anti-mitotic activity to colchicine but which does not disrupt microtubules (Grisham, Wilson & Bensch, 1973), had a variable, low-level inhibitory effect which was not concentration-dependent over the range  $5\text{--}40 \mu\text{g/ml}$  (Figs. 3, 4). The effects of lumicolchicine and griseofulvin were similar for both the lectin-induced and the spontaneous cases. None of these drugs had any effect on patching.

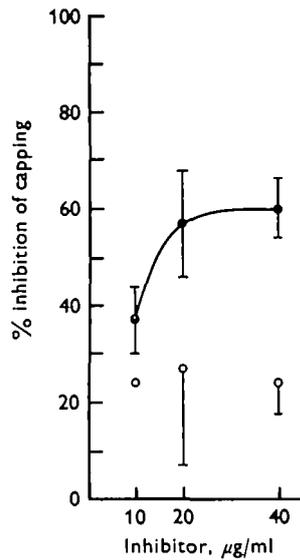


Fig. 3

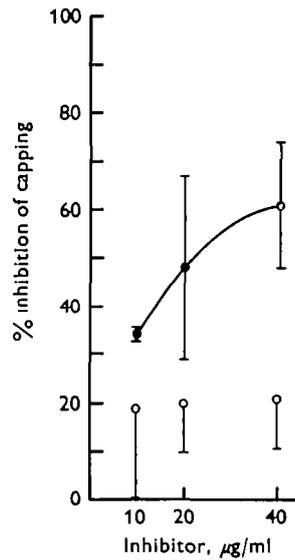


Fig. 4

Fig. 3. Inhibition of lectin-induced cap formation of Con A receptors by colchicine (●) and griseofulvin (○).

Fig. 4. Inhibition of spontaneous cap formation of Con A receptors by colchicine (●) and griseofulvin (○).

Both cytochalasin B (CB) and cytochalasin E (CE) had an inhibitory effect on cap formation and no effect on patch formation. In the presence of glucose (HBSG) CB inhibited both induced and spontaneous capping by 72% at a concentration of  $20 \mu\text{g/ml}$  (Figs. 5, 6). On the other hand, CE inhibited capping by only 40% under both conditions. DMSO had no effect on redistribution. CB has been shown to inhibit hexose transport by as much as 80% compared to 20% for CE (Kletzien, Perdue & Springer, 1972; J. F. Perdue, personal communication). To avoid complications due to these different effects on transport the experiments were repeated in the absence of glucose (HBS, pH 7.2). Under lectin-induced conditions CB inhibited capping by 40% (versus 70% in HBSG) while the effect of CE was unchanged. Replacement of glucose with 2-deoxyglucose ( $2 \text{ mg/ml}$ ) resulted in a level of inhibition similar to that achieved in the presence of glucose. Taken together, these data suggest that glucose or its analogue 2-deoxyglucose in some way facilitates the effect of CB. This may be

related to the existence of multiple binding sites for CB, one of which seems to be associated with the glucose transport site (Lin & Spudich, 1974).

Treatment with colchicine (40  $\mu\text{g/ml}$ ) or CB (20  $\mu\text{g/ml}$ ) did not reverse the inhibition of capping due to high concentrations of lectin as has been observed with lymphocytes (Unanue & Karnovsky, 1974; Edelman, Yahara & Wang, 1973).

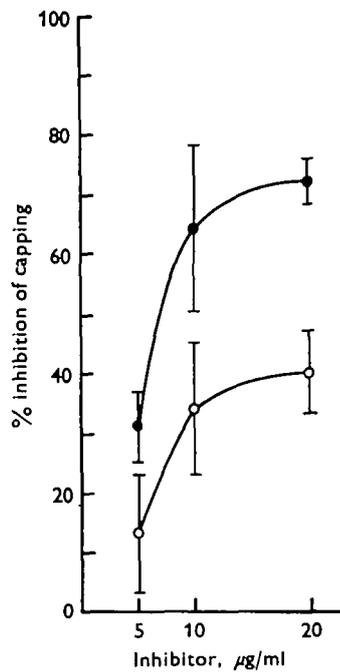


Fig. 5

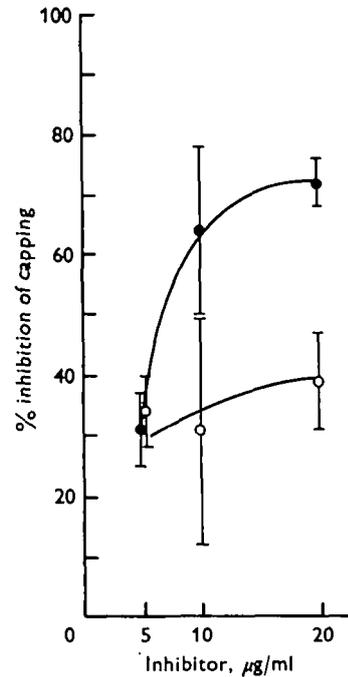


Fig. 6

Fig. 5. Inhibition of lectin-induced cap formation of Con A receptors by cytochalasins B (●) and E (○), as assayed in HBSG.

Fig. 6. Inhibition of spontaneous cap formation of Con A receptors by cytochalasins B (●) and E (○), as assayed in HBSG.

#### *Method of cell preparation and surface receptor distribution*

Cells prepared by mechanical dispersion were found to have a consistently higher percentage of the population in the ring configuration initially than cells prepared by trypsinization (Table 2). In addition, mechanically dissociated cells formed very few caps (2–4 %) under either lectin-induced or spontaneous conditions but did show a moderate level of patching (30 %) after 60 min at 22 °C under either condition. Trypsinization of mechanically dissociated cells (4000 units/ $8 \times 10^7$  cells, 15 min at 37 °C) followed by lectin-induced redistribution conditions resulted in the same extent of redistribution as was found for trypsin-dissociated cells.

The observation that mechanically dispersed cells do not undergo an extensive surface redistribution of receptors while cells prepared by trypsinization do, suggests that trypsin modifies the cell surface in some way, allowing both spontaneous and lectin-induced redistributions to occur. If this were the case, cellular repair processes

might be expected to overcome the effect of trypsinization. To test this idea, trypsin-dissociated cells were plated in 60-mm bacteriological culture dishes ( $2 \times 10^7$  cells/dish) in complete Eagle's medium with 1X non-essential amino acids and an extra 2 mg/ml glucose and cultured for 1–4 h in an atmosphere of 10% CO<sub>2</sub> in air. At various

Table 2. *Effects of mechanical dispersion, trypsinization, and incubation time in trypsin solution on the initial distribution of Con A receptors on freshly dispersed cells*

	Time in dissociation medium, min	No. of cells with ring fluorescence, % $\pm$ s.e.
Trypsin	10	81 $\pm$ 4
	20	67 $\pm$ 7
	30	61 $\pm$ 7
CMF 0.1% EDTA	30	86 $\pm$ 3
	30	85 $\pm$ 5

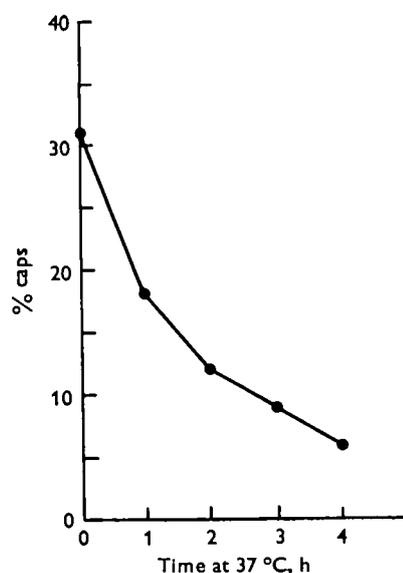


Fig. 7. Loss by trypsin-dispersed cells of the capability to redistribute surface Con A receptors into caps during 4 h in culture at 37 °C. At each time point  $20 \times 10^6$  cells were washed twice in HBSG and assayed for the degree of lectin-induced cap formation after 60 min as described in Materials and methods.

time points, the cells were harvested, washed twice with HBSG, and assayed for the extent of lectin-induced redistribution as described in Materials and methods. The results of this experiment are shown in Fig. 7. By the end of 4 h in culture, trypsin-dissociated retina cells are no longer able to redistribute their Con A receptors into caps to any significant degree.

The effect of trypsin on initial receptor distribution does not vary with concentration between 1000 and 16000 units/ml, but varying the amount of time in the trypsin

solution at any one trypsin concentration affects initial receptor distribution. The longer the period of incubation in trypsin the fewer the cells with a ring distribution immediately following dispersion (Table 2). A 10-min incubation at 37 °C in either calcium- and magnesium-free Tyrode's solution or in complete Tyrode's solution prior to trypsinization had no effect on the percentage of cells initially in a ring configuration.

#### *Lectin-mediated agglutination of cells*

Con A-mediated agglutinability of neural retina cells prepared by trypsinization appears to be independent of the ability to redistribute surface receptors into patch or cap configurations. Cells with maximal numbers of rings, patches or caps were prepared by incubation in HBSG for 0, 30 or 60 min, respectively. These cells were tested for agglutinability by Con A at concentrations of 0.15 to 33 µg/ml either directly or after fixation for 30 min with 2 % glutaraldehyde as described in Materials and methods. All of these preparations were agglutinated similarly, 3 µg/ml of Con A being the least amount giving positive agglutination. In addition, cells prepared by trypsinization and allowed to repair in culture for 4 h, a time sufficient to abolish their capacity for cap formation, were also agglutinable by 3 µg/ml Con A. Identical results were obtained for WGA-mediated agglutination. It is clear from these results that major redistributions of lectin receptors which occur either prior to or during the assay do not influence agglutinability. However, this does not rule out the possibility that 'micro' redistributions not detectable with fluorescence light microscopy play an important role in agglutination.

Mechanically dispersed cells showed some spontaneous flocculation in the absence of added lectin but did not show any enhanced agglutination with Con A at concentrations as high as 100 µg/ml. Neither live nor fixed mechanically dissociated cells were agglutinated in this assay.

#### *Receptor distribution in whole tissues*

In order to examine the distribution of Con A receptors on cells in an intact tissue, whole retinas were labelled with FITC-Con A before or after fixation. Small pieces of the tissue were then squashed carefully beneath a coverslip on a slide and examined for the distribution of fluorescent label. The permeability of whole tissues to lectin was sufficient to allow most cells to display bright ring fluorescence. Tissues fixed immediately after dissection but not trypsinized, or fixed immediately following trypsinization and then labelled with FITC-Con A, showed only ring fluorescence on individual cells. In addition, cells in intact, trypsinized tissues did not undergo a spontaneous redistribution of Con A receptors when assayed for 60 min in HBSG. In tissues not treated with trypsin, no lectin-induced redistribution was observed. However, cells in trypsinized, intact tissues were able to undergo a receptor redistribution into patches and caps when incubated at 22 °C under lectin-induced conditions. These results suggest that lectin receptors remain stationary in the plane of the membrane in intact tissues even in the presence of ligand. Following trypsin treatment, but not dispersal into single cells, lectin-induced redistribution can occur. Dispersal into a single cell population is apparently necessary for any spontaneous redistribution to occur.

## DISCUSSION

Our results demonstrate that surface lectin receptors on single chick neural retina cells prepared by trypsinization are able to undergo a spontaneous rearrangement in the plane of the membrane in the absence of exogenously added multivalent ligand as well as a more extensive rearrangement after pre-incubation with lectin. Mechanically dissociated cells, on the other hand, are able to undergo only limited redistribution of surface receptors under either spontaneous or lectin-induced conditions. Similarly, cells in intact tissues not subjected to trypsinization do not undergo either spontaneous or lectin-induced redistributions.

The maximum degree of capping found on cells prepared by trypsinization is 31 % under lectin-induced conditions. The question must be raised whether this represents a specific sub-population of cells capable of forming caps or, alternatively, whether the figure is a true measurement of the capacity for cap formation in the entire cell population. The repair experiment offers some insight into this problem. As Fig. 7 shows, after 60 min at 37 °C there is a loss of approximately 40 % in the number of cells having caps. It might therefore be expected that during the 60-min incubation at 22 °C during which redistribution is monitored, there would be progressive repair of the trypsin damage which limits the number of cells which are able to form caps. Presumably, in the absence of both continued protein synthesis and endogenous pools of surface component precursors the degree of capping would be greater. Experiments to examine this possibility are currently in progress.

As has been reported for lymphocytes and fibroblastic cell lines (de Petris, Raff & Mallucci, 1973; Inbar & Sachs, 1973), we have found that capping is unaffected by cycloheximide and significantly inhibited by sodium azide or high concentrations of lectin while patching is unaffected by any of these treatments. In addition, we have shown that capping is inhibited by both colchicine and cytochalasin B. Both of these drugs have been used extensively in studies on cell surface receptor mobility. It has been found, for example, that cytochalasin B inhibits capping but not patching of Con A receptors on 3T3 cells (de Petris *et al.* 1973) and, to a lesser extent, capping of surface immunoglobulin on lymphocytes (Unanue & Karnovsky, 1974; Edelman *et al.* 1973). Colchicine, on the other hand, has been found to have no effect on patching or capping in lymphocytes but does reverse the inhibition of capping due to high lectin concentration (Unanue & Karnovsky, 1974; Edelman *et al.* 1973).

Although the details of the drug effects on receptor mobility may vary with cell type, microtubules and microfilaments seem to be involved in all cases. Patch formation appears to be a passive lateral diffusion process while cap formation is energy-dependent. In addition, capping seems to be under at least 2 separate control systems; one intracellular and mediated through microtubules and microfilaments, and the other extracellular and due to cell surface interactions with ligands in the external milieu. These 2 systems may be interconnected (Edelman *et al.* 1973); however, in our experiments this did not appear to be the case since treatment with colchicine or cytochalasin B did not reverse the inhibition of capping caused by high concentrations of lectin.

It has been suggested that lectin-mediated agglutination requires lateral mobility of

receptors in the plane of the membrane (Rutishauser & Sachs, 1974). The observation of Inbar *et al.* (1973) that fixed cells are unable to agglutinate supports this contention. However, in our experiments, cells prepared by dissociation and fixation agglutinated almost as readily as unfixed cells. This result suggests that if lateral mobility is required for agglutination its role may be to provide a 'micro' redistribution of surface receptors. If this is the case, such a distribution is achieved by trypsin treatment alone in our system, and once such a micro redistribution has occurred, fixation does not abolish agglutinability. In this light, it is somewhat paradoxical that mechanically dissociated cells do not agglutinate since they do undergo patch formation, albeit to a lesser extent than cells prepared by trypsinization. Patch formation may represent a passive 'diffusion' process, while capping is indicative of an underlying capacity of cells to redistribute surface components in a directed manner, and agglutinability may also require directed movement of surface receptors. On the other hand, it is possible that mechanically dissociated cells are not agglutinable because of some unique property of their surface which results from the dissociation process itself. The cells may be carrying intercellular material or fragments of membrane from adjacent cells on their surfaces. This interpretation is suggested by the finding that 4-h cultured cells are agglutinable in spite of the fact that they share with mechanically dissociated cells a very low capacity for cap formation. The low degree of capping displayed by mechanically dissociated cells *per se* does not account for the lack of agglutinability of these cells.

We have been unable to repeat earlier results (Kleinschuster & Moscona, 1972) that mechanically dissociated chick neural retina cells are agglutinable by Con A. However, in their experiments the lowest concentration of lectin used was 50  $\mu\text{g}/\text{ml}$ , and dose-dependent increases in the size of agglutinates were scored rather than the limiting dilution of lectin necessary for agglutination, as was done in our experiments. The results with mechanically dissociated cells are further complicated by the spontaneous self-flocculation these cells exhibit in the absence of lectin and the difficulty in obtaining single cells. It is likely that particular conditions of dissociation and fixation can radically alter the behaviour of cells in agglutination assays, and in this case meaningful comparison of results may not be possible.

Redistribution of a fluorescently labelled ligand on a cell surface is usually interpreted to be a reflexion of the fluid nature of the plasma membrane itself as expected from consideration of the fluid mosaic model of membrane structure (Singer & Nicolson, 1972). If this is the case, then our results seem to bear more generally on the nature of the cell membrane of cells *in situ* versus single cells or cells dissociated from tissues. Immobility of surface receptors on cells in intact tissues has not been reported previously and may indicate that once cells are incorporated into compact or condensed tissues the plasma membrane becomes 'locked' in a specific configuration. It may be significant, in this regard, that cells prepared by trypsinization progressively lose their ability to undergo lectin-induced cap formation over time in culture. Since these cells originate from a partially differentiated tissue, repair processes in culture may return the cell surface to a state more similar to that which exists *in situ*; that is, a state in which the cells have decreased membrane fluidity due to their incorporation

into a tissue mass. This further suggests that membrane fluidity may be an important parameter during the early processes of tissue formation when extensive cell movements are occurring. This idea is also consistent with our recent observation that factors which mediate cell recognition and adhesion obtained from cultures of embryonic chick neural retinas or cerebral lobes inhibit cap formation in a tissue-type specific manner (McDonough & Lilien, 1975).

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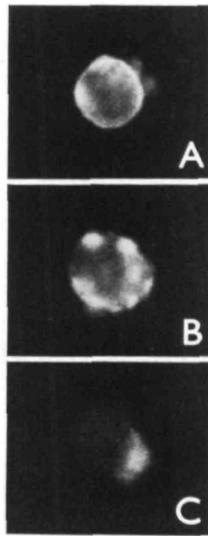


Fig. 8. Representative photomicrographs of cells having ring (A), patch (B), and cap (C) fluorescence.  $\times 1285$  approx.