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# AN INTRACELLULAR POOL OF A CELL-SURFACE LIGAND WHICH INHIBITS LECTIN-INDUCED CAPPING

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## SUMMARY

It has previously been established that cells dissociated from embryonic chick neural retina tissues redistribute their cell surface Concanavalin A receptors into patches and caps upon treatment with the lectin. During 4 h in continuous culture the cell population progressively loses the capacity to cap Con A receptors. In the presence of cycloheximide this decrease occurs for only the first 2 h of incubation. Concomitant with this loss in the ability to redistribute Con A receptors, cells become agglutinable by a tissue-specific component first described in 1975.

The results of our experiments assaying inhibition of capping by tissue culture supernatant solutions and loss of capping ability through cellular repair processes are interpreted in terms of an endogenous pool of one or more species, which, when displaced to the cell surface, inhibit the capping of Con-A receptors and permit tissue-specific cell agglutination. Once present at the cell surface, these species are released into the culture medium, either during continuous culture or during an incubation in fresh medium containing cycloheximide. Subsequent to release of these species, the cells regain their capacity for cap formation and lose agglutinability. In addition, the components released from the cells inhibit capping of Con A receptors when added to freshly dissociated cells.

The assay methods were also used to determine parameters governing mobilization of the pool of capping inhibitors to the cell surface.

## INTRODUCTION

Tissue culture medium conditioned by embryonic chick neural retinae contains macromolecular components which inhibit the lectin-induced redistribution of cell surface receptors into caps on freshly dissociated neural retina cells (McDonough & Lilien, 1975*a*). The capping-inhibition activity is tissue-type specific: medium obtained from neural retina cultures has no effect on capping of lectin receptors on single cerebral lobe cells and vice versa. In addition, for neural retina, the capping inhibition activity is lost upon treatment of the medium with $\beta$-*N*-acetyl hexosaminidase, but not with pronase (Lilien & Rutz, 1977).

Capping of plant lectin receptors among single cells is also inhibited through intrinsic cell processes which occur during *in vitro* culture (McDonough & Lilien, 1975*b*). In this paper, assays for inhibition of capping by tissue culture supernatant solutions and loss of capping ability through cellular repair processes are exploited to establish the existence of an endogenous pool of the tissue-type specific capping inhibitor. The assays are also used to determine the parameters which govern mobilization of the pool to the cell surface.

Neural retina tissue culture conditioned media also contain a macromolecular species which binds at the cell surface and renders the cells competent to be agglutinated by a tissue specific component which accumulates in serum free monolayer conditioned media (Lilien & Rutz, 1977; Balsamo & Lilien, 1974a). The acquisition of agglutinability by cells fixed after various incubation regimes indicate that a pool of the species which renders cells agglutinable also exists, and is indistinguishable from that of the capping inhibitor. The data suggest that a single molecular species which inhibits capping and is required for agglutination is found both as a pool in cells and free in tissue culture supernatants. We will refer to this species as 'ligand', although it should be kept in mind that both activities need not reside in one molecule.

## MATERIALS AND METHODS

### *Cell preparation*

*Freshly trypsinized cells.* Neural retinae from 10-day white leghorn chick embryos were obtained by dissection in warm Tyrode's solution (pH 6·5). Groups of 3 retinae were placed in 10-ml Erlenmeyer flasks, washed once with 3 ml of calcium- and magnesium-free Tyrode's solution (CMF), and incubated for 10 min at 37 °C in 3 ml of CMF on a rotating platform (40 rev/min). The tissues were washed with warm Hepes (0·01 M) buffered saline with added glucose (1 mg/ml, HBSG, pH 7·2) and then incubated with rotation for 20 min at 37 °C in 3 ml of HBSG (pH 7·4) containing 4000 National Formulatory Units/ml of trypsin (Miles, 3x crystalline). Following 3 washings in 3 ml of warm Tyrode's solution (pH 6·5) the tissues were dissociated in 3 ml of warm Tyrode's solution containing 50 μg/ml of deoxyribonuclease (NBC, crystalline) by flushing through a Pasteur pipette with the tip flamed to an opening of 0·5 mm. The resulting cell suspension was counted utilizing a particle counter (Coulter Electronics), pelleted at 200 g for 5 min at 4 °C and washed once with ice-cold HBSG.

*Cultured cells.* Cells prepared by the above procedure were cultured at 37 °C in Eagle's Basal Medium containing an additional 2 mg/ml glucose, 2 % non-essential amino acids, 1 mM glutamine, and 50 μg/ml gentamycin (complete medium) in 60-mm Falcon bacteriological dishes ($2 \times 10^7$ cells/4 ml/dish) under a moist atmosphere of 10 % $CO_2$ in air. At the times indicated, cells were collected from the dishes by flushing with a Pasteur pipette, pelleted for 5 min at 300 g at 4 °C, and washed once with ice-cold HBSG containing 5 μg/ml of cycloheximide (Sigma) (HBSG-CH). This concentration of the drug is sufficient to reduce [$^3$H]-leucine incorporation into trichloroacetic acid-precipitable material by 95 % within 10 min.

### *Assay for redistribuion of Con A receptors*

Cell suspensions were assayed for the extent of capping exhibited by individual cells by methods described previously (McDonough & Lilien, 1975 *a, b*) with the following modifications: 0·1 ml of cells suspended in HBSG-CH ($2{\cdot}5 \times 10^7$ cells/ml) was added to 0·05 ml of fluorescence-labelled Concanavalin A (FITC-Con A, 100 μg/ml in HBSG, Miles) and 0·4 ml of HBSG containing various additives. Following 10 min at 5 °C, the samples were incubated for 60 min at 37 °C, pelleted for 5 min at 200 g at 5 °C, and resuspended in 0·4 ml of 2 % glutaraldehyde in 0·02 M sodium phosphate (pH 7·4) at 5 °C. The proportion of the cell population exhibiting cap fluorescence was determined using a Zeiss Universal microscope modified for epifluorescence.

### *Release of cell surface macromolecules*

Cell surface macromolecules with the capacity to inhibit lectin-induced cap formation of Con A receptors were released from cells by resuspending washed cells at a concentration of approximately $10^8$ cells/ml in HBSG-CH, incubating for 10 min at 37 °C, and then pelleting for 5 min at 200 g at 5 °C and washing once with ice-cold HBSG-CH. The cells were then either assayed for Con A receptor redistribution as described above or returned to culture.

To collect the material released by the 10-min incubation in HBSG-CH (release period), the cell suspension was pelleted for 10 min at 200 **g** at 5 °C. The supernatant was collected and recentrifuged for 10 min at 10000 **g**. The high-speed supernatant (released material, RM) was then dialysed overnight at 5 °C against 0·01 M sodium-phosphate-buffered saline (pH 7·4). Protein concentration in the RM was determined by the method of Lowry, Rosebrough, Farr & Randall, 1951) using crystalline bovine serum albumin as a standard. The capping inhibition activity of the RM was determined by incubating various concentrations of the dialysed released material in 0·5 ml of HBSG-CH containing freshly dissociated neural retina cells as described above.

To assess the importance of terminal sugar residues for RM activity, 1·0 ml of RM was added to 0·5 ml of 0·05 M sodium acetate buffer (pH 4·2) containing 0·033 units/ml of purified $\beta$-*N*-acetyl hexosaminidase (Miles, *Turbo cornutus*) and the mixture incubated for 30 min at 30 °C. The reaction was terminated by boiling for 10 min and the digested RM was then dialysed overnight against phosphate-buffered saline (pH 7·4) and assayed for inhibition of capping activity as above. Boiled controls lacking enzyme were done at the same time and retained more than 95 % of the activity of the untreated RM.

### *Fixed cell agglutination assay*

Cells pretreated in various ways were fixed in 2 % glutaraldehyde (Ladd, EM grade) in 10 mM sodium phosphate-buffered saline (0·12 M NaCl: pH 7·4) for 30 min at 4 °C. The cell suspension was then pelleted at 1000 **g** for 5 min at 4 °C, resuspended in 0·2 M glycine in 10 mM sodium phosphate (pH 7·4), and incubated for 10 min at 22 °C. Following 4 washes with 0·15 M NaCl – 0·01 M sodium phosphate (pH 7·4), aliquots of $10^6$ cells/3 ml/35-mm Falcon dish were incubated at 70 rev/min for 24 h in serum-free medium conditioned by monolayers of 10-day neural retina cells. Agglutination was scored by Coulter Counter determination of the number of single cells remaining.

Monolayers were prepared by aliquoting $20 \times 10^6$ freshly dissociated cells to 35-mm Falcon plastic tissue culture dishes in 4·0 ml of complete medium. After 24 h the medium was collected, centrifuged at 10000 rev/min for 3 min and made 2 mM with phenyl methyl sulphonyl fluoride in isopropanol.

## RESULTS

### *Characterization of the endogenous pool of inhibition of capping activity*

*Utilization of the endogenous pool.* The majority of freshly dissociated 10-day chick neural retina cells display the ability to redistribute their surface Con A receptors into caps (Fig. 1). During 4 h in culture this ability is progressively lost (Fig. 1, open circles). However, when cycloheximide (5 $\mu$g/ml) is included in the culture medium the loss of capping proceeds only for approximately 2 h with a return to nearly the initial level of freshly dissociated cells after a total of approximately 4 h in culture (Fig. 1, filled circles). A possible interpretation of these observations is that: (1) trypsinization removes some component (or components) from the cell surface which functions to restrict cell surface receptor mobility; (2) there exists an endogenous pool of this material which is mobilized to the cell surface during repair in culture; and (3) in the presence of cycloheximide, the mobilized pool material is spontaneously released from the cell surface into the surrounding medium. By operationally defining the pool as the pre-existing supply of a component which limits the re-distribution of lectin receptors into caps and which is not functionally localized at the cell surface following single cell preparation, it has been possible to explore the above interpretation.

The capacity to cap Con A receptors may also be restored by washing and incubating

the cells for 10 min at 37 °C in fresh medium containing cycloheximide (designated release period) following an initial culture period. This result suggests that the cell surface components inhibiting capping are either released into the medium or otherwise inactivated by this treatment. That the former hypothesis is correct is shown by experiments in section II. Whichever hypothesis is correct, it is possible to verify the presence of an endogenous pool by monitoring changes in the capping capacity of cells recultured in cycloheximide after a release period. The size of the endogenous

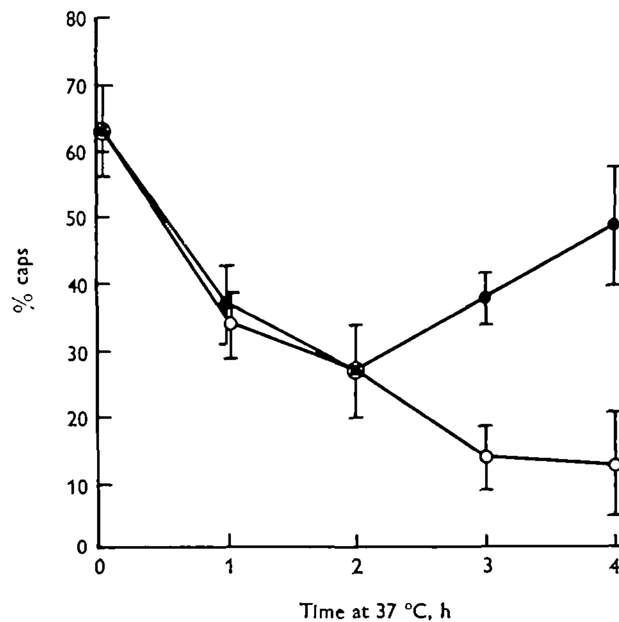


Fig. 1. Loss of the capacity for capping of Con A receptors by 10-day chick neural retina cells during continuous cell culture. At each time point an aliquot of cells was harvested, washed in ice-cold HBSG-CH, and assayed for its extent of capping. Values shown are mean ± S.D. from 2–4 separate experiments. ●, ○, incubation medium with and without 5 $\mu$g/ml cycloheximide, respectively.

pool can be estimated by determining the length of time during the initial culture period in cycloheximide in which a reduction in the capacity to cap Con A receptors occurs.

As shown in Fig. 2, cells recultured after an initial 4 h at 37 °C in medium containing cycloheximide and a subsequent 10-min release period do not lose their capacity for capping during an additional 2 h in medium containing cycloheximide. If cycloheximide is omitted during the reculture period the cells do show a progressive loss of the capacity for cap formation. Considering the data presented in Figs. 1 and 2 together, it appears that freshly dissociated cells contain a pool of capping inhibitory activity which becomes progressively utilized during approximately the first 2 h in culture. Thus, reculture in the presence of cycloheximide following a release period does not result in any detectable loss in these cells' capacity for capping. The loss in the ability of cells to cap Con A receptors seen during reculture in the absence of

cycloheximide must therefore be due to the rapid utilization of newly synthesized material which inhibits capping.

It is apparent from Fig. 2 that the endogenous pool is depleted during the initial 4 h at 37 °C whether or not cycloheximide is included in the medium since reculture in the presence of cycloheximide following a 10-min release period results in no loss in the ability to cap Con A receptors. Thus, it seems that some event during single cell preparation – perhaps trypsinization or dissociation – triggers the cells to begin

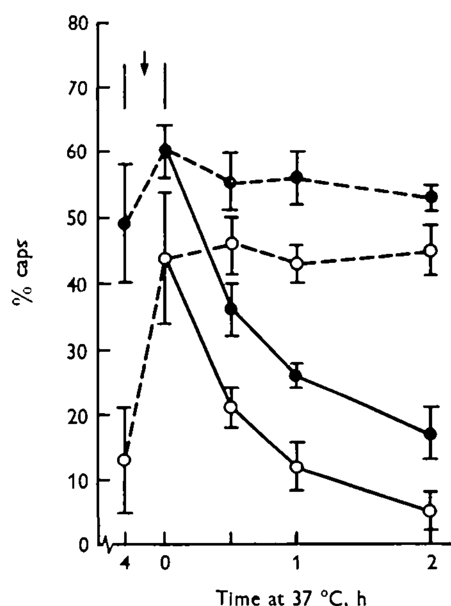


Fig. 2. Capacity of cells to undergo Con A-induced capping during continuous cell culture. After 4 h of continuous culture with (●) or without (○) cycloheximide (5 μg/ml) the cells were harvested, washed once with ice-cold HBSG-CH and then incubated for 10 min at 37 °C in HBSG-CH at a concentration of approximately $10^8$ cells/ml (release period). The cells were then pelleted, washed with ice-cold HBSG-CH and returned to culture at 37 °C with (dashed line) or without (solid line) cycloheximide (5 μg/ml). At each time point an aliquot of cells was harvested, washed in ice-cold HBSG-CH, and assayed for its extent of capping. Values shown are means ± S.D. from 2–4 separate experiments.

utilizing their internal pool independently of whether or not *de novo* synthesis is occurring (see Discussion). Furthermore, the pool is replaced in culture only over long time periods. After 24 h in culture approximately 1 h of pool has been reconstituted.

*Effects of temperature on pool utilization.* Throughout 4 h at 5 °C the percentage of the harvested cells which can cap is essentially identical whether or not cycloheximide is included in the medium. Culture for 4 h at 22 °C results in a progressive loss of capping the rate of which is intermediate between that occurring at 37 and 5 °C.

These data suggest that the rate of pool displacement is temperature-dependent and imply that after 4 h at 5 or 22 °C the cells still contain some proportion of their pool

material. Therefore, the release period-reculture protocol described above was utilized to determine the amount of pool material remaining in cells cultured at the two temperatures. In spite of the fact that culture at 5 °C results in a slight decrease in the cells' capacity for capping during the 4-h culture period, following this period the entire endogenous pool still remains in these cells as measured by this assay (Fig. 3). Similarly, while cells cultured at 22 °C appear to mobilize most of their endogenous pool to the cell surface during a 4-h incubation period, they still contain a portion of their pool when assayed after the initial incubation (Fig. 3).

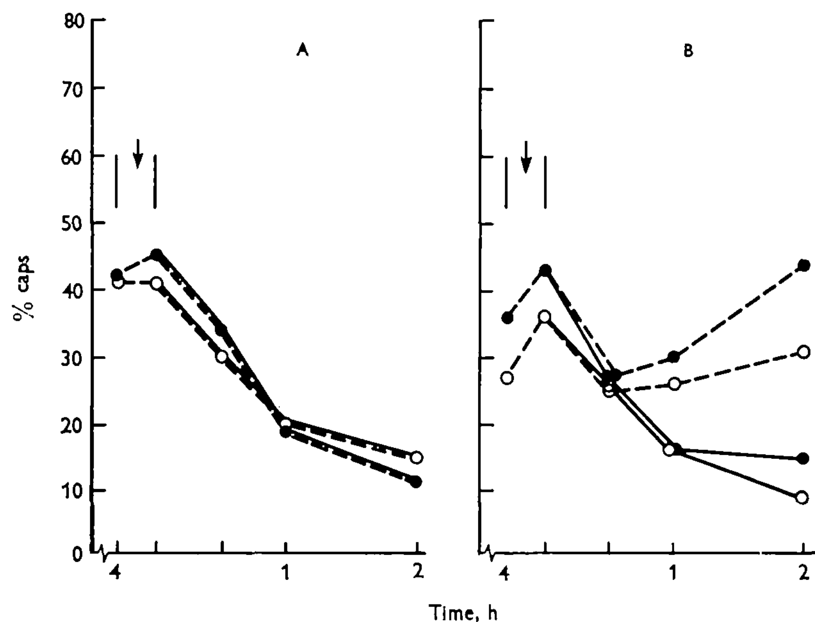


Fig. 3. Effects of incubation at different temperatures on loss of capacity for capping of Con A receptors by 10-day retina cells in continuous cell culture. Conditions are as described in the legend to Fig. 2, except that the initial 4-h incubation was done at either 5 °C (A) or 22 °C (B). Values shown are means from duplicates from 2 separate experiments. ●, ○, incubation medium with and without cycloheximide (5 μg/ml), respectively. Following the release period, cells were incubated with (dashed lines) or without (solid lines) cycloheximide (5 μg/ml) at 37 °C.

*Effects of drugs on pool utilization.* The effects of various compounds on pool utilization were tested by including them in the culture medium during the first 4-h incubation at 37 °C. The cells were then harvested and recultured following a release period to determine the amount of pool remaining. The results of these experiments are shown in Table 1. Incubation in medium containing cycloheximide and either colchicine, cytochalasin B, sodium azide, or retina tissue culture supernatant solutions (Balsamo & Lilien, 1974*b*, 1975) results in the cells' retaining their entire pool, indicating that mobilization of the pool has been completely inhibited. On the other hand, treatment with cytosine arabinoside does not hinder complete pool utilization. In all of these experiments controls were done which contained inhibitor but which were preincubated at 4 °C instead of 37 °C. These provided an

estimate of background inhibition of capping of Con A receptors due to the inhibitors themselves. This is necessary as the effects of colchicine and azide are not completely reversible under these conditions (McDonough & Lilien, 1975*b*).

Table 1. *Effects of additives on pool utilization*

| Treatment* | Size of pool remaining (h) after 4 h in culture† |
|---|---|
| None | 0 |
| Colchicine, 20 μg/ml | 2·0 |
| Cytochalasin B, 40 μg/ml | 2·0 |
| $NaN_3$, 2·5 mM | 2·0 |
| Retina tissue culture supernatants, 20 μg/ml‡ | 2·0 |
| Cytosine arabinoside, 5 mM | 0 |
| 4 °C | 2·0 |
| 22 °C | 0·5 |

* Additives were included in the culture medium and cells were cultured for 4 h in the presence of cycloheximide at 37 °C except as indicated. At the end of the incubation the cells were harvested, washed, incubated under conditions that promote release of cell surface material as described in the legend to Fig. 2, and recultured at 37 °C (in all cases) with or without cycloheximide.

† The size of the pool remaining after the first 4-h incubation was assayed in terms of the length of time that cells recultured in the presence of cycloheximide continued to show a decreased capacity for capping.

‡ Retina tissue culture supernatants were collected from cultures of whole 10-day chick neural retinas as described by McDonough & Lilien (1975*b*).

*Age-dependent changes in pool size.* We have used the time point at which cultures containing cycloheximide begin to regain the capacity to cap Con A receptors as a measure of the relative pool size of different preparations. There is a clear age-dependent difference in the pool size when retinas of 7-, 10-, and 13-day chick embryos are allowed to repair in culture with and without cycloheximide. The time required to deplete the pool in the presence of cycloheximide decreases with increasing developmental age. Relatively undifferentiated 7-day retina cells have approximately a 3-h pool while the more differentiated 13-day retina cells have only a 30-min pool as determined by this assay. The rates of decrease in the percentage of cells with caps whether in the presence or absence of cycloheximide are not significantly different for the three ages.

### *Activity of the released material*

The previous results are all consistent with either release of the mobilized material or its inactivation at the cell surface. To distinguish between these alternatives the released material (RM) was collected and assayed for its ability to inhibit capping. The results of these experiments are shown in Table 2. It may be seen that, as predicted, only the RM collected after a 10-min release period in cycloheximide has activity in the assay. In addition, this activity is tissue-type specific; capping

inhibition is seen upon testing with retina cells but not with cerebral lobe cells. Freshly dissociated retina cells or cells incubated for 2 h in drugs that inhibit utilization of the pool (cytochalasin B, $NaN_3$) do not release material that inhibits capping. Again, these results confirm the dynamics of pool utilization described earlier and suggest that material active in inhibiting capping is not released into the medium directly from the pool, but rather must first be functionally mobilized to the cell surface. It is interesting that the supernatant from continuous 2-h cultures incubated in the presence of cycloheximide is inactive in this assay while the supernatant from 4-h cultures is active. This may indicate that the major portion of the pool is displaced to the cell surface before any detectable amount is released into the medium via turnover processes.

Table 2. *Capping inhibition activity of released material and culture supernatants*

| Preparation | Activity |
|---|---|
| Freshly trypsinized + 10-min release period | − |
| 2 h at 37 °C with cycloheximide (CH), 5 μg/ml: | |
| Culture supernatant* | − |
| 4 °C wash supernatant* | − |
| 10-min release period | + |
| 2 h at 37 °C + CH + cytochalasin B (40 μg/ml) + 10-min release period | − |
| 2 h at 37 °C + CH + $NaN_3$ (2·5 mM) + 10-min release period | − |
| 4 h at 37 °C + CH* culture supernatant* | + |

Samples were assayed for capping inhibition activity at 37 °C. Released material was considered active if it inhibited capping of Con A receptors by more than 50 % at a concentration of less than 30 μg/ml. Retina aggregation-promoting supernatants yield greater than 50 % inhibition of capping at a concentration of 5 μg/ml and maximally inhibit capping by 80 % at a concentration of 25 μg/ml (see McDonough & Lilien, 1975*a*). Conditions for release of cell surface macromolecules were as described in the legend to Fig. 1.

* Culture or wash supernatants were collected after centrifugation at 4 °C for 5 min at 200 g and dialysed overnight at 5 °C against 0·15 M NaCl–0·01 M sodium phosphate (pH 7·2).

Retina tissue culture conditioned media contain glycoproteins which inhibit capping of lectin receptors (McDonough & Lilien, 1975*a*) and bind specifically to neural retina cells (Balsamo & Lilien, 1974*b*, 1975). Both activities depend on the presence of a terminal *N*-acetyl galactosamine residue in the saccharide moiety. Therefore, an analysis of the activity of the released material from cells cultured for 2-h in medium containing cycloheximide was carried out after treatment with purified *N*-acetyl-hexosaminidase. When tested for inhibition of capping, the digested RM had lost 73 % of its activity.

### *Fixed-cell agglutination assays*

As shown in Table 3, the data obtained by agglutination assays are in agreement with the results described above. Cells preincubated 2 h in cycloheximide, harvested,

and fixed show maximal agglutination; after an additional 10-min release period agglutination is reduced to the level of fresh, trypsin-dispersed cells. In addition, cells incubated for 2 h in cycloheximide containing either cytochalasin B, colchicine, or sodium azide and then fixed show reduced agglutination. Thus, during cell culture in the presence of cycloheximide, material appears at the cell surface which both restricts capping and mediates cell agglutination. Furthermore, compounds which inhibit mobilization of the endogenous pool material also inhibit the appearance at the cell surface of macromolecules which mediate agglutinability.

Table 3. *Agglutinability of fixed cells*

| Treatment during culture for 2 h at 37 °C | Release period (RP) | % of maximal agglutination |
|---|---|---|
| None | No RP | 100 |
| | RP | 65 |
| + cycloheximide (CH, 5 μg/ml) | No RP | 98 |
| | RP | 20 |
| + CH + cytochalasin B, 40 μg/ml | No RP | 35 |
| | RP | 38 |
| + CH + colchicine, 20 μg/ml | No RP | 28 |
| | RP | 38 |
| + CH + $NaN_3$, 2·5 mM | No RP | 33 |
| | RP | 39 |
| + CH + cytosine arabinoside, 2 mM | No RP | 84 |
| | RP | 35 |
| Fresh trypsin-dispersed cells | — | 27 |

Results are the average of 3 separate experiments.

## DISCUSSION

These results suggest that there exists in embryonic chick neural retina cells an endogenous pool of one or more ligands which can be mobilized to the cell surface in the presence of cycloheximide. Once incorporated into the surface, these ligands are able both to inhibit cap formation of Con A receptors and to mediate agglutination of fixed cells. The ligands are removed from the cell surface either through cellular turnover processes occurring during 4 h of culture in medium containing cycloheximide or through a more rapid process occurring during an incubation of as little as 10 min in HBSG-CH (release period). In either case, it appears that only that portion of the pool which has been mobilized to the cell surface can be released into the medium. The released ligands which originate in the endogenous pool are tissue-type specific for inhibition of capping. Furthermore, the cells, after release of ligands from their surfaces, regain the capacity for cap formation and lose agglutinability.

It appears that cells maintain a complement of the ligand on their surfaces through interactions among pool, cell surface, and medium components. It is clear from the experiments in which retina tissue culture supernatants cause inhibition of pool

utilization that some feedback from the cell surface to the pool must occur. Thus, the existence of surface-localized ligand supplied exogenously and possibly endogenously restricts utilization of the pool. Furthermore, synthesis of new cell surface ligand may be at least partially controlled by the existence of unreleased pool material. As shown in Fig. 1, even in the absence of cycloheximide, the pool appears to be utilized (open circles) without concomitant replacement. This may indicate that in the presence of the pool *de novo* synthesis is inhibited and only when most of the pool has been depleted and released can synthesis and utilization of new material occur.

We have no direct evidence which pertains to the cellular localization of the pool. While these data are consistent with a model involving insertion into the plasma membrane and lateral redistribution of an intracellular pool (Baudiun, Stock, Vincent & Grenier, 1975; Redman, Banerjee, Howell & Palade, 1975), they cannot distinguish between this mechanism and the possibility of rearrangements of a cryptic cell surface-localized ligand pool.

Several lines of evidence imply that the capping-inhibitory component and the surface ligand which mediates agglutinability are identical. As reported herein the dynamics of pool utilization and release appear to be identical. In addition, tissue culture-conditioned media contain both capping inhibitory activity (McDonough & Lilien, 1975*a*) and a component which when fixed at the cell surface renders them agglutinable (Lilien & Rutz, 1977; Balsamo & Lilien, 1974*a*). Both activities are tissue-type specific and labile to $\beta$-*N*-acetyl hexosaminidase, as is the released material. Whether or not a single species is found to mediate both activities, the profound effects of these molecules on cell behaviour and membrane architecture make them prime candidates for roles as critical determinants of morphogenetic interactions.

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