

# EFFECTS OF CYCLIC AMP AND SEPHADEX FRACTIONS OF CHICK EMBRYO EXTRACT ON CLONED RETINAL PIGMENTED EPITHELIUM IN TISSUE CULTURE

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## ABSTRACT

The effects of dibutyl cyclic 3',5'-adenosine monophosphate (BcAMP) and Sephadex G-25 fractions of chick embryo extract on the growth rate, morphology, and pigmentation of normal chick retinal pigmented epithelium (PE) were investigated. Seven cloned PE cell lines were each grown in modified Ham's F-12 medium alone (F-12), or in F-12 supplemented with either high molecular weight (H) or low molecular weight (L) fractions of chick embryo extract. Cells grown in F-12 alone or in L medium formed compact epithelial sheets, whereas cells grown in H had a fibrocytic appearance and formed poorly organized monolayers. In H plus BcAMP, cell morphology was more epithelioid than in H alone, and generally the monolayers appeared more differentiated. Under each of these three culture conditions,  $2 \times 10^{-4}$  M BcAMP retarded the increase in cell number and decreased the final number of cells per culture dish, but had little effect on plating efficiency. BcAMP also increased the rate of cell adhesion to a plastic substratum. Pigmentation was marked in cultures grown in F-12 or in L alone, but the addition of BcAMP dramatically reduced visible pigmentation. This effect was reversed when BcAMP was removed from the culture medium. Thus BcAMP modifies cell and colonial morphology, rate of cell accumulation, adhesive properties, and pigmentation of normal PE cells.

## INTRODUCTION

Dibutyl cyclic 3',5'-adenosine monophosphate (BcAMP) has been shown to elicit a more "normal" morphological appearance in cultured transformed cell lines (1, 2) and to decrease the rate of cell accumulation in cultures of transformed cells (3, 4), normal fibroblasts (5, 6), and epidermal cells (7, 8). Cyclic AMP and its derivatives have also been reported to increase pigmentation in cultured melanoma cells (9) and have been implicated in the process of pigment granule transloca-

tion within dermal and epidermal melanocytes of the frog (10, 11, 12). The effects of cyclic nucleotides on normal pigmented neuroepithelial cells, however, have not been investigated.

This communication reports the effects of cyclic AMP and BcAMP on plating efficiency, attachment rate, cell and colonial morphology, cell accumulation, and pigmentation of cloned populations of normal embryonic chick retinal pigmented epithelial cells (PE) grown in culture media that either promote or discourage differentiation. Our studies reinforce the notion that BcAMP is involved in contact inhibition of cellular proliferation. Moreover, BcAMP promotes a more differentiated morphology, increases the rate of attachment to a plastic substratum, and has an unexpected inhibitory effect on the development of visible pigmentation in these normally melanin-synthesizing cells.

## MATERIALS AND METHODS

### *Cell Techniques*

Embryos of *Gallus domesticus* were staged according to the criteria of Hamburger and Hamilton (13). Donor eyes were placed in 0.25% trypsin solution (Grand Island Biological Co., Grand Island, N. Y.), and posterior pole PE was dissected cleanly. The epithelial sheets were rinsed in saline G (14), and dissociated in Coon's enzyme solution (CTC: 6 U/ml collagenase, 0.1% trypsin, 2% chick serum) containing 4 mM EDTA (CTC-EDTA). Single pigmented cells were selected from this suspension with a fine glass pipette, and plated at low density in 60-mm plastic tissue culture dishes (Falcon Plastics, Division of B-D Inc., Los Angeles, Calif.) in Coon's modification of Ham's F-12 medium (F-12) containing 5% fetal calf serum and 0.3  $\mu$ g of penicillin/ml (15). Resultant clonal colonies were isolated and serially propagated by the usual techniques. Cell lines were routinely maintained in 3 ml of F-12/dish in a 5% CO<sub>2</sub>, 95% air atmosphere (100% humidity) at 37.5°C. The medium was changed every 3 days.

### *Embryo Extract*

High (H) and low (L) molecular weight fractions of chick embryo extract were prepared on a Sephadex G-25 column according to the method of Coon and Cahn (15) and added to F-12 medium (containing 5% fetal calf serum) to form the "H medium" and the "L medium," respectively. Embryo extract was added to achieve a final concentration of 5% (vol/vol), taking the volume of the complete embryo extract sample applied to the column as the original volume of each fraction.

### *Culture Methods*

Replicate experimental plates were established for each of seven PE cell lines by adding a known number of cloned PE cells to 3 ml of H medium or L medium. When appropriate,  $2 \times 10^{-4}$  M BcAMP (Sigma Chemical Co., St. Louis, Mo.), purified according to the method of Falbriard et al. (16), was added to the culture medium. Control dishes consisted of the same number of cells in H or L medium without BcAMP. The medium was changed every 2 days. The number of cells per dish was determined by suspending two dishes of the cells from each experimental condition in CTC-EDTA solution and counting with a hemocytometer at 24-h intervals for at least 8 days.

### *Cell Adhesion Determination*

Cells in the post log phase of growth were incubated for 1 h in the presence or absence of 1 mM BcAMP, suspended in CTC-EDTA, pelleted by centrifugation at 600 *g* for 4 min, and resuspended in F-12. Cells were counted in a hemocytometer and dispersed into 60-mm plastic tissue culture dishes containing 3 ml F-12 that had been allowed to equilibrate to 37.5°C. Then 0.1 ml BcAMP (0.1 ml of water for the controls) was added to appropriate dishes, and all dishes were incubated for from 5 to 80 min. At the end of the incubation period, the medium was removed, the plates were gently washed twice with saline G to remove nonadherent cells, and 1.0 ml of CTC-EDTA solution was added. After a 20-min incubation, cells were quantitated by duplicate hemocytometer counts. Three separate experiments were performed in this manner, two with cells derived from chick embryos at stage 23 of development, one with cells from stage 34. The number of cells plated was varied among experiments, and was either 1.2, 2.5, or  $7.0 \times 10^5$  cells/dish.

### *Microscope Techniques*

Morphology was observed and documented through a phase-contrast microscope fitted with a 35 mm camera. Pigmentation in the PE monolayers grown under various conditions was estimated by measuring the optical density of a 1-cm diameter circle of confluent cells with a densitometer (Photovolt Corp., New York). Density was expressed logarithmically in arbitrary units.

All fixation and embedding procedures for electron microscopy were performed in the culture dishes without manipulation of the colonies, in order to preserve spatial relationships. Cultures were briefly rinsed in one to three changes of saline G and were fixed either in 1% osmium tetroxide (0.14 M Veronal acetate, pH 7.3) at 0°C for 15 min, or in 1.5% glutaraldehyde (0.05 M sodium cacodylate, 4% sucrose,

pH 7.2) for 15 min at room temperature, and then for another 15 min at 4°C, followed by postfixation in 1% osmium tetroxide (0.15 M phosphate buffer, pH 7.2) for 1 h at 4°C. An ethanol series was used for dehydration, and either Spurr epoxy resin (Polysciences Inc., Warrington, Pa.) or Epon 812 (Shell Oil Co., New York) was used for embedding. Representative areas of the monolayers were carefully selected by phase-contrast microscopy of the embedded cells. Thin sections of these areas were cut with glass or diamond knives on an LKB Ultratome III (LKB Instruments, Rockville, Md.) or a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were stained with uranyl acetate and lead citrate by standard methods, and examined with a JEM-100B electron microscope.

### Phosphodiesterase Assay

Cyclic nucleotide phosphodiesterase was determined at 25°C by the method of Thompson and Appleman (17).

## RESULTS

### Plating Efficiency

The plating efficiencies for the clones of PE cells in H and L media with and without cyclic AMP or BcAMP are shown for stage 23 embryos in Table I. Similar data were obtained in five other experiments with cells from stages 25, 26, and 27. The presence of BcAMP had little effect on plating efficiency in either H or L medium. In each case, cells in the H medium attached to the culture dish in greater numbers than in the L medium.

### Cell Adhesion

Cells, seeded into medium containing 1 mM BcAMP at the time of plating, consistently showed an increased rate of adhesion to the substratum compared to cells in medium not containing BcAMP irrespective of whether they had or had not been preincubated with 1 mM BcAMP (Fig. 1). Cells which were preincubated with BcAMP, but did not receive BcAMP at the time of plating, showed only slightly greater adhesiveness than cells never exposed to BcAMP. At the end of each experiment, the total recovery of cells (those removed in the wash solution plus those removed from the plate by CTC-EDTA treatment) agreed well with the original number of plated cells. In separate experiments, the addition of 1 mM theophylline per dish was found to greatly decrease

TABLE I  
*Plating Efficiency and Number of Cells Per Culture Dish 5 Days Post Log Phase for Cells from Stage 23 Embryos*

Growth medium, drug	Plating efficiency	Number of cells per dish (10 <sup>6</sup> )
	%	
L, no drug	29	1.07
L, cAMP	30	1.15
L, BcAMP	33	0.95
H, no drug	37	2.00
H, cAMP	39	1.85
H, BcAMP	35	1.00
F-12, no drug	27	1.60
F-12, cAMP	25	1.48
F-12, BcAMP	30	1.05

$2.0 \times 10^{-4}$  M cyclic nucleotide (BcAMP) was used. Cell numbers were averages of two plates of cells grown under each experimental condition. Agreement of duplicates was within 10%.

cell adhesion and, in general, to be deleterious to cell growth and morphology.

### Growth Rate

Retinal PE cells cultured in H medium increased in number more rapidly than cells in L medium (Fig. 2). Cells grown in H medium with 0.2 mM BcAMP, however, proliferated more slowly than in H medium alone and at approximately the same rate as cells grown in L medium (Fig. 2). The addition of BcAMP to L medium did not significantly affect growth rate. Similar results were seen with 1 mM BcAMP.

All dishes to which BcAMP was added contained fewer cells 5 days after the cessation of log phase growth than those without BcAMP (Table I). Cells grown in H medium containing BcAMP showed this effect most markedly. Theophylline, added to cells cultured in either H or L medium, markedly inhibited cell proliferation.

The addition of cyclic AMP to H medium resulted in a slight but definite decrease in the rate of accumulation of the PE cells, as well as a slightly smaller final number of cells per dish.

### Phase-Contrast Morphology

By 7 days after plating, the majority of the PE cells grown in L medium had formed typical

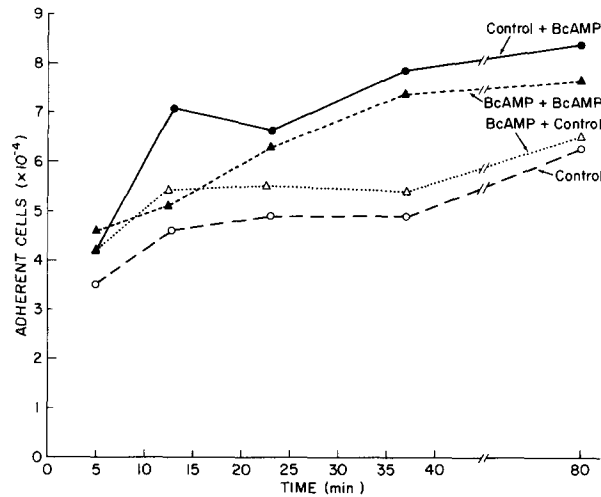


FIGURE 1 Effect of BcAMP on cell adhesion. Cells were preincubated for 1 h with or without 1 mM BcAMP ("BcAMP" or "control") followed by washing and incubation from 5 to 80 min in the presence or absence of 1 mM BcAMP. Control (○—○) received no BcAMP; BcAMP + control (△····△) received BcAMP only in the preincubation period; BcAMP + BcAMP (▲---▲) received BcAMP in both preincubation and incubation periods; control + BcAMP (●—●) received BcAMP only in the incubation period. Values given are averages of duplicate hemocytometer counts from each of four replicate cultures. Values agreed within 10%.

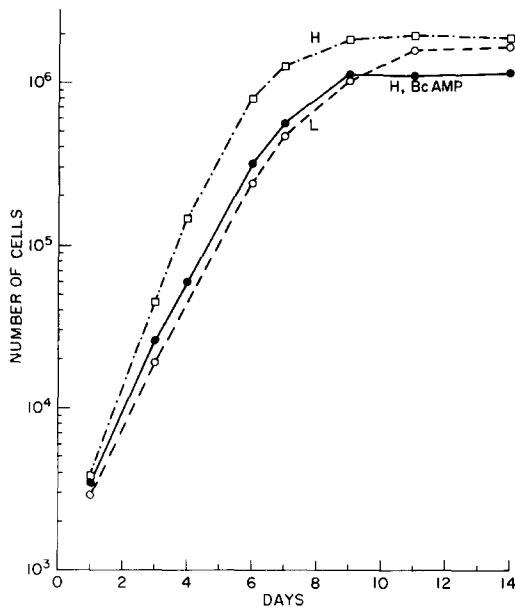


FIGURE 2 Effect of BcAMP on growth rate. Cells were grown in H medium (□---□), L medium (○---○), or L medium containing  $2 \times 10^{-4}$  M BcAMP (●---●). Values given are averages from four replicate culture dishes; values agreed within 10%.

colonies which were discrete and rounded with relatively smooth, regular boundaries, and which consisted of compact, regularly packed cells. Individual cells displayed considerable differentia-

tion. They had similar polygonal shapes, distinct boundaries, and moderate pigmentation (Fig. 3 A).

Most cells from the same PE clone that were grown in H medium, in contrast, did not form distinct colonies, but were spread irregularly throughout the culture dish. Those colonies that were formed usually had irregular boundaries which suggested substantial cell movement into and out of the colonies. Individual cells in these colonies were usually stellate or spindle shaped, lightly pigmented, and had less distinct outlines than cells in colonies grown in L medium (Fig. 3 B).

Cells of the same clone grown in H medium plus BcAMP formed more colonies than those grown in H alone. These colonies appeared as lightly pigmented as those grown in H alone, but were generally more distinct and more rounded. Their peripheries were more regular, which suggested less cell movement (Fig. 3 C). Individual cells in most of these colonies were less spread and had more clearly visible cell outlines than cells in H medium alone. Thus, with respect to colony morphology and cell differentiation, cells grown in H plus BcAMP appeared to be intermediate between the cells grown in H and those grown in L.

Ruffled plasma membranes were observed in cells grown under all three conditions but were

particularly striking in those grown in H medium alone. In L medium and in H plus BcAMP, ruffling was confined to the periphery of the colonies, whereas in H medium it often occurred within the colonies (Figs. 3 and 4).

Cells observed after 70 days of growth in L medium were confluent and appeared well differentiated. They could be distinguished easily from those grown in H medium by their smaller sizes, regular polygonal shapes, distinct boundaries, and greater pigment content (Fig. 4).

### *Ultrastructure*

After 70 days of growth in L medium, PE cells typically had a cuboidal form and exhibited distinct polarity (Fig. 5). The apical regions had extensive and regularly positioned intercellular junctions of the zonula adherens type. The basal regions had a continuous basal lamina and a uniform layer of extracellular fibrillar material which included some strands with the size and macroperiodicity of native collagen. The boundaries between cells were oriented approximately perpendicular to the surface of the tissue culture plate. The cell nuclei were spheroid, and they tended to be basally located. Golgi apparatuses were seen most often in central to apical positions. Premelanosomes and melanosomes were abundant.

Cells grown for 70 days in H medium appeared fibroblastic. They had numerous intercellular junctional complexes, but these were not regularly positioned. The cells were squamous rather than cuboidal, and both the cells and their nuclei were flattened in the plane of the tissue culture plate (Fig. 6). The cell boundaries were oblique rather than normal to the surface of the tissue culture plate. An increased amount of extracellular filamentous material was observed. No basal lamina was present, but a substance which had an electron density and appearance similar to that of basal lamina filled many cisternae of the endoplasmic reticulum and occurred in copious amounts extracellularly (Fig. 6 B). The extracellular filamentous material was not distributed uniformly along the cell surface as it was in cells grown in L medium. Strikingly fewer premelanosomes or other pigment bodies were found.

After only 9 and 17 days of growth, fewer ultrastructural differences were observed between cells grown in L medium and those grown in H medium than were apparent after 70 days of growth in these media. Cells grown in L medium were not cuboidal after 17 days of growth, although they

did have a greater apex-to-base dimension than cells grown in H medium. More of the cells grown in L medium were tightly adjoined to their neighbors by tortuous junctional regions than were cells grown in H medium (compare Figs. 7 and 8). The cell boundaries in L medium tended to be more vertically oriented, on the average, than those in H medium. The most obvious difference was that cells grown in L medium had many premelanosomes and melanosomes, while those grown in H medium had only an occasional pigment body. The cells in L medium had formed a well-defined basal lamina and supporting layers of fibrillar material which were continuous in most of the colonies (Fig. 7), whereas those in H medium had no basal lamina and had fibrillar layers which were uneven and were disrupted more frequently (Fig. 8).

Cells grown for 9 and 17 days in H medium plus BcAMP had many intercellular junctional complexes, well-developed endoplasmic reticulum, and abundant extracellular filamentous material, some of which had the appearance of segments of basal lamina and some of which appeared to be native collagen (Fig. 9). The cells grown in H medium plus BcAMP were similar to those grown in H medium alone, in that they had essentially no pigment bodies. They were intermediate between cells grown in L medium and those grown in H medium, in that their boundaries were usually more tortuous and more vertically oriented than those grown in H medium alone, and that at least segments of basal lamina were identifiable, suggesting a more epithelioid character.

### *Visible Pigmentation*

PE cells grown in L medium invariably exhibited more visible pigmentation than companion cells grown for the same length of time in F-12 medium (Figs. 10 A and B). After approximately 14 days in culture, PE cells grown in H medium usually developed faint but definite visible pigmentation. Regardless of the medium, growth in the presence of BcAMP was associated with a virtual absence of visible pigmentation (Figs. 10 D, E, and F). The development of pigmentation was both delayed and reduced in the presence of BcAMP, as revealed by optical density measurements (Fig. 11). This effect was reversible. When BcAMP was no longer added to the medium, pigmentation increased to nearly the amount present in cells not exposed to this exogenous nucleotide

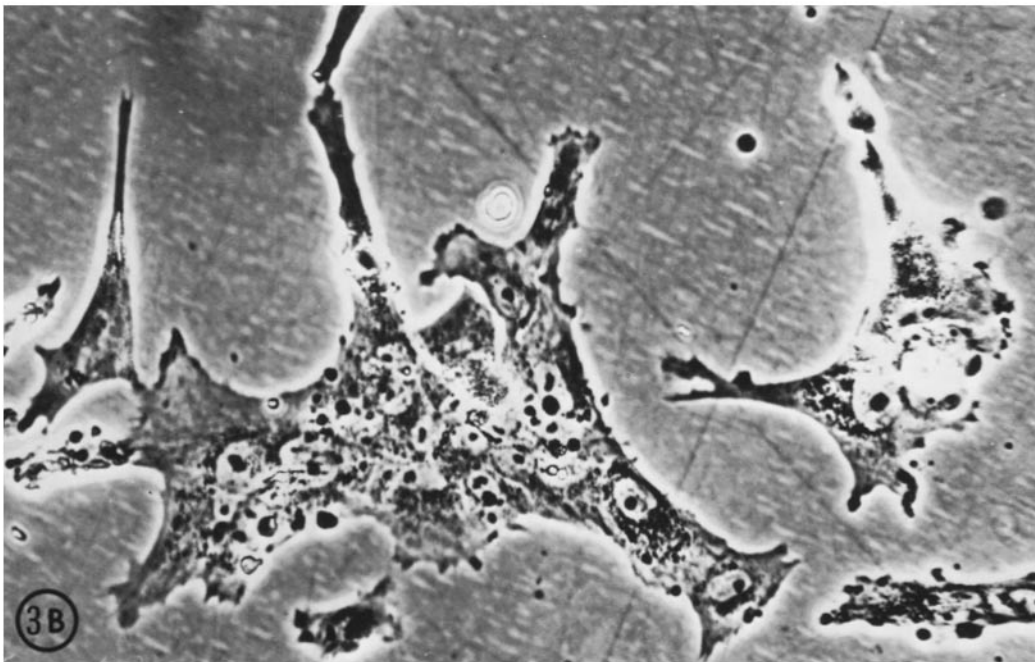
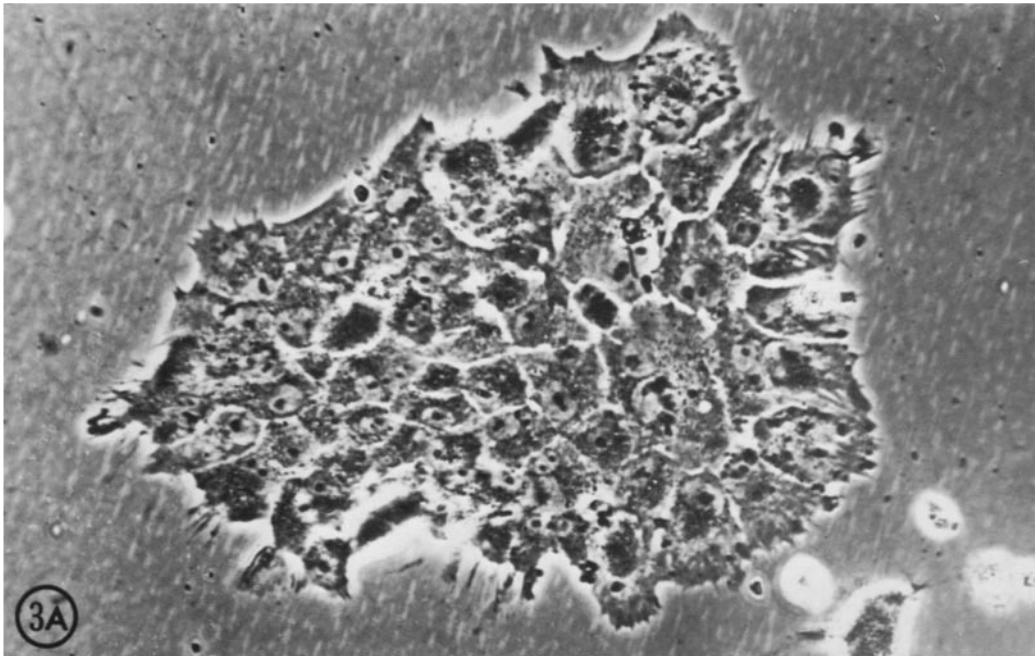
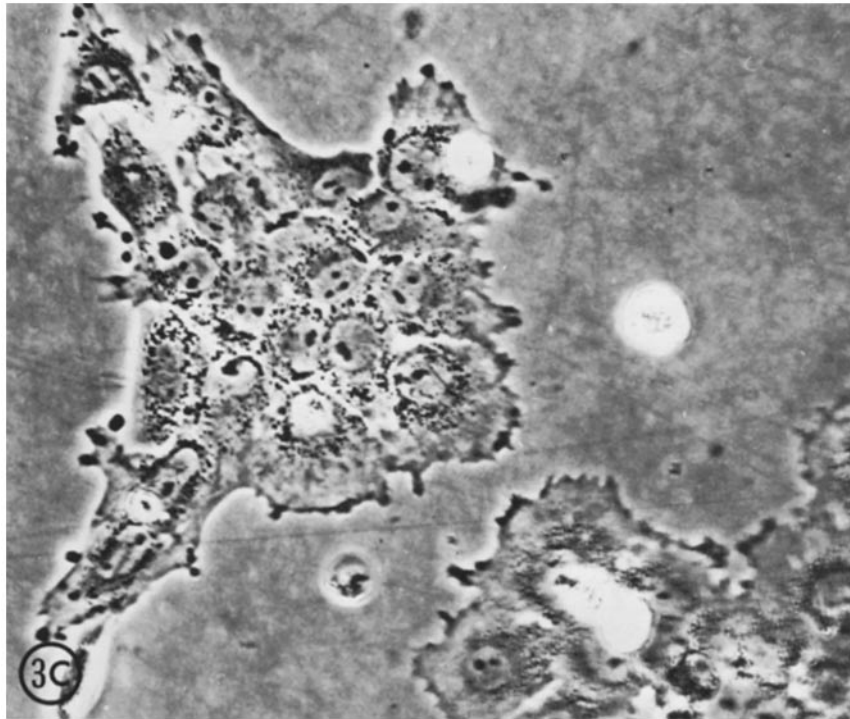


FIGURE 3 Pigmented epithelial cells cloned from stage 26 chick embryos passed through three serial passages, then cultured for 7 days. (Fig. 3 A) Ham's F-12 medium plus the light molecular weight fraction of embryo extract (L); (Fig. 3 B) F-12 medium with the heavy molecular weight fraction of embryo extract (H); and (Fig. 3 C) F-12 medium with H plus 1 mM dibutyryl cyclic AMP.  $\times 645$ .



(Fig. 11). Cyclic AMP produced similar but less marked effects.

Although the enhancement of pigmentation in L medium was observed consistently for each of the seven clones used in this study, the degree of enhancement definitely varied from clone to clone and from one batch of medium to the next.

#### *Phosphodiesterase Determinations*

The H medium, containing the protein fraction of the chick embryo extract and also containing calf serum, exhibits significant cyclic nucleotide phosphodiesterase activity, 49 pmol cyclic AMP hydrolyzed/ml/min. Phosphodiesterase activity in L medium was found to be approximately 15-fold lower; this activity can probably be ascribed to contamination of the L fraction of embryo extract by the H fraction.

#### **DISCUSSION**

Cells from the same cloned line of embryonic chick retinal PE can be made to appear well differentiated or poorly differentiated by the respective addition of either the L or H fraction of embryo extract to the growth medium (15). In

the present study, cells grown in H medium were poorly differentiated. They were characterized by a fibrocytic rather than an epithelioid appearance, decreased deposition of pigment, increased membrane ruffling, increased rate of cell accumulation, and higher saturation density. Thus by growing normal cells in the H medium, we were able to elicit many of the characteristics of transformed cell lines *in vitro*. When BcAMP was added to the H medium, total cell population and saturation density were lowered to levels seen in L medium; the cells exhibited more epithelial characteristics including less ruffling activity of the plasma membranes.

It now seems fairly certain that cyclic AMP and its derivatives slow the rate of cellular proliferation and influence the saturation density properties of normal and transformed cells (3, 5, 18). The present study indicates that these effects of cyclic AMP also apply to normal neuroepithelial cells *in vitro*, in contrast to the reported lack of such effects in a variety of transformed epithelial cell lines (2). The mechanism by which cyclic AMP exerts these effects is not known. Retinal PE cells grown in H medium, however, may be analogous in their characteristics to trans-

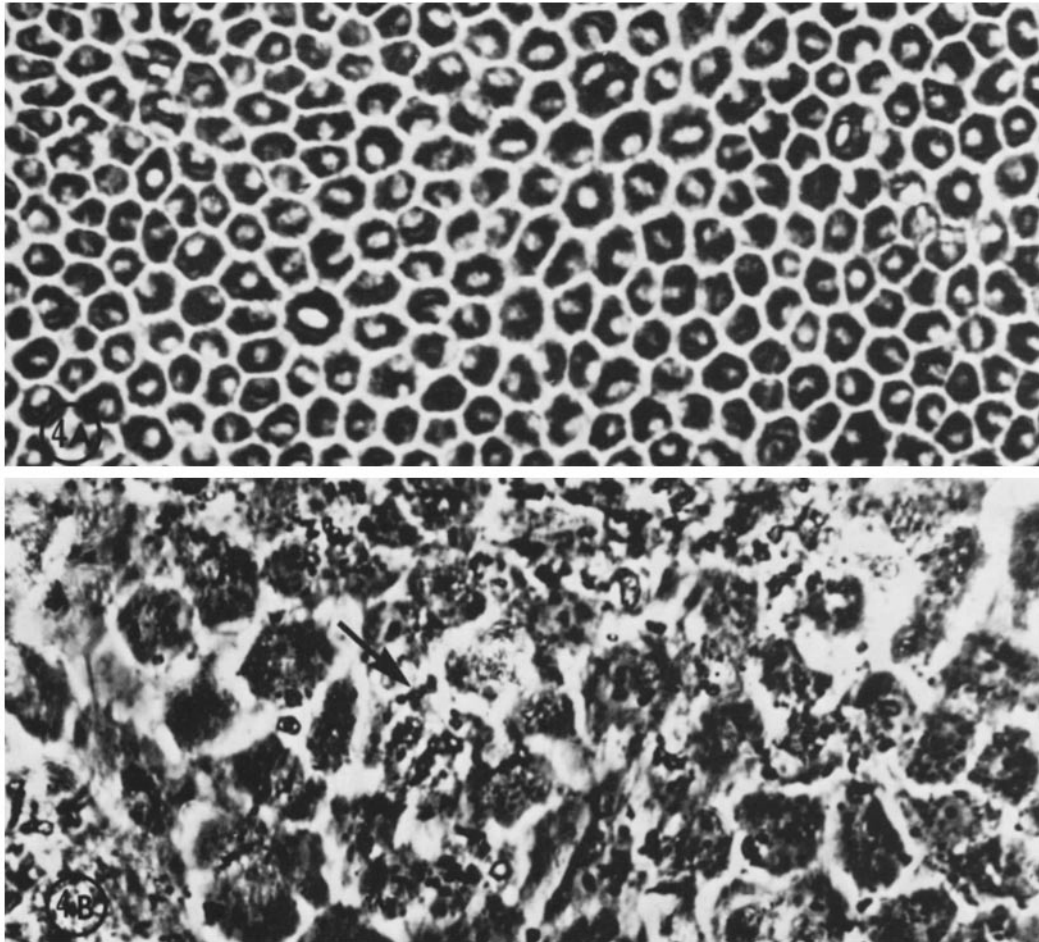


FIGURE 4 Pigmented epithelial cells from the same clone (stage 26 donor), cultured for 70 days in (Fig. 4 A) L medium; or (Fig. 4 B) H medium (arrow indicates region of membrane ruffling).  $\times 420$ .

formed cells which have lower cyclic AMP levels than their normal (nontransformed) counterparts. Medium containing the H fraction of chick embryo extract may be inhibitory in some way to adenylate cyclase or other factors which regulate cyclic AMP levels.

The final plating efficiency of cells under a variety of culture conditions was not influenced by the presence of either cyclic AMP or BcAMP. The initial rate of cell attachment was enhanced, however, by the presence of BcAMP. This effect, whether due to the elaboration of surface components or to other factors such as cell flattening or decreased motility, is a rapid one. Cells exposed to BcAMP for only 5–10 min exhibited significant changes in attachment characteristics. The

rapidity of this effect and the fact that preincubation of cells for 1 h with BcAMP had only a small effect on attachment, would indicate that these changes do not depend on *de novo* synthesis of a new factor. These results are in accord with those of Johnson and Pastan (19) who found that cells treated with BcAMP were more resistant to removal from their substratum, but can be contrasted with results of Grinnell et al. (20) who found that BcAMP did not affect the rate at which transformed or normal baby hamster kidney cells attached to a substratum.

Little is known about the control of melanogenesis in vertebrates. Johnson and Pastan (9) have demonstrated that cyclic AMP and BcAMP influence the morphology of cultured melanoma



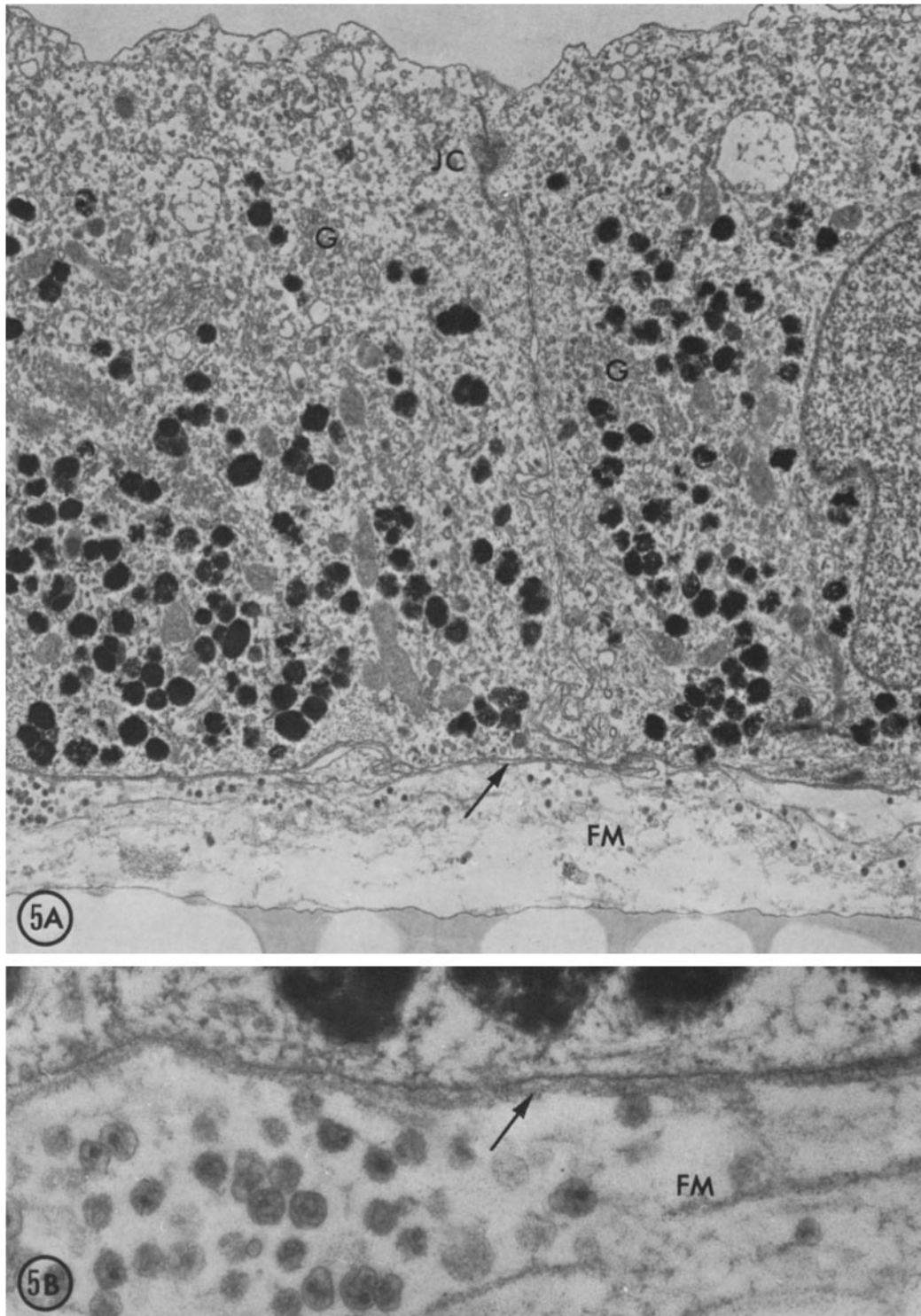


FIGURE 5 Transsections of pigmented epithelial cells from the same clone seen in Fig. 4 but cultured for 70 days in L medium: (Fig. 5 A) typical cells showing intercellular junctional complexes (*JC*), Golgi (*G*), basal lamina (arrow), and extracellular filamentous material (*FM*), as well as abundant pigment granules.  $\times 12,000$ . (Fig. 5 B) Basal region of a cell showing a well-developed basal lamina and some filamentous material.  $\times 48,000$ . The extracellular particles are viruses which are commonly seen in cultures of chick cells.

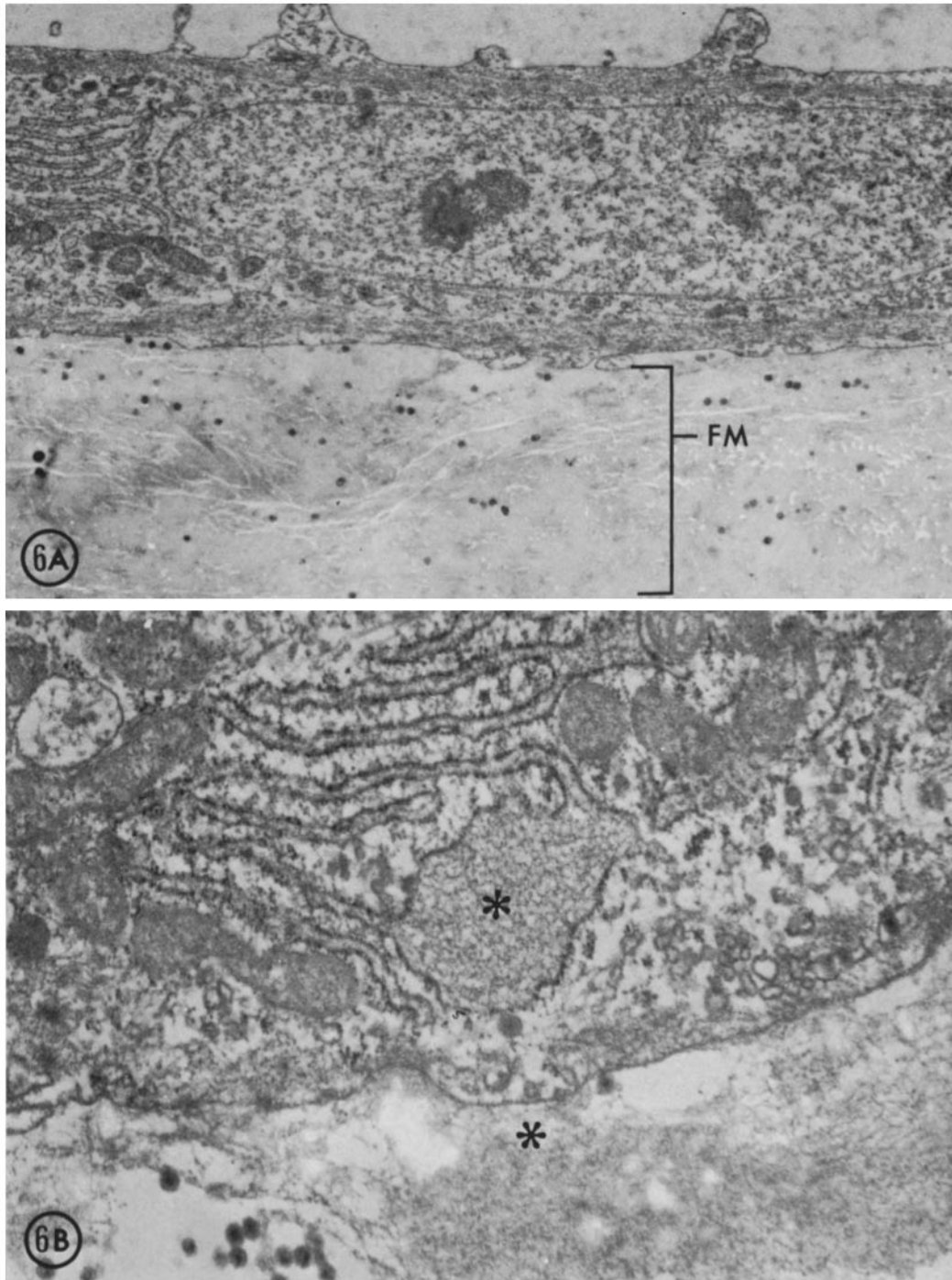


FIGURE 6 Transections of cells from the same clone as those in Fig. 5, but cultured for 70 days in H medium: (Fig. 6 A) typical cell showing flattened nucleus and abundant extracellular filamentous material (*FM*).  $\times 12,000$ . (Fig. 6 B) Basal region of a cell showing absence of a basal lamina, but copious amounts of a substance which has a similar morphology both intracellularly and extracellularly (asterisks).  $\times 38,000$ .

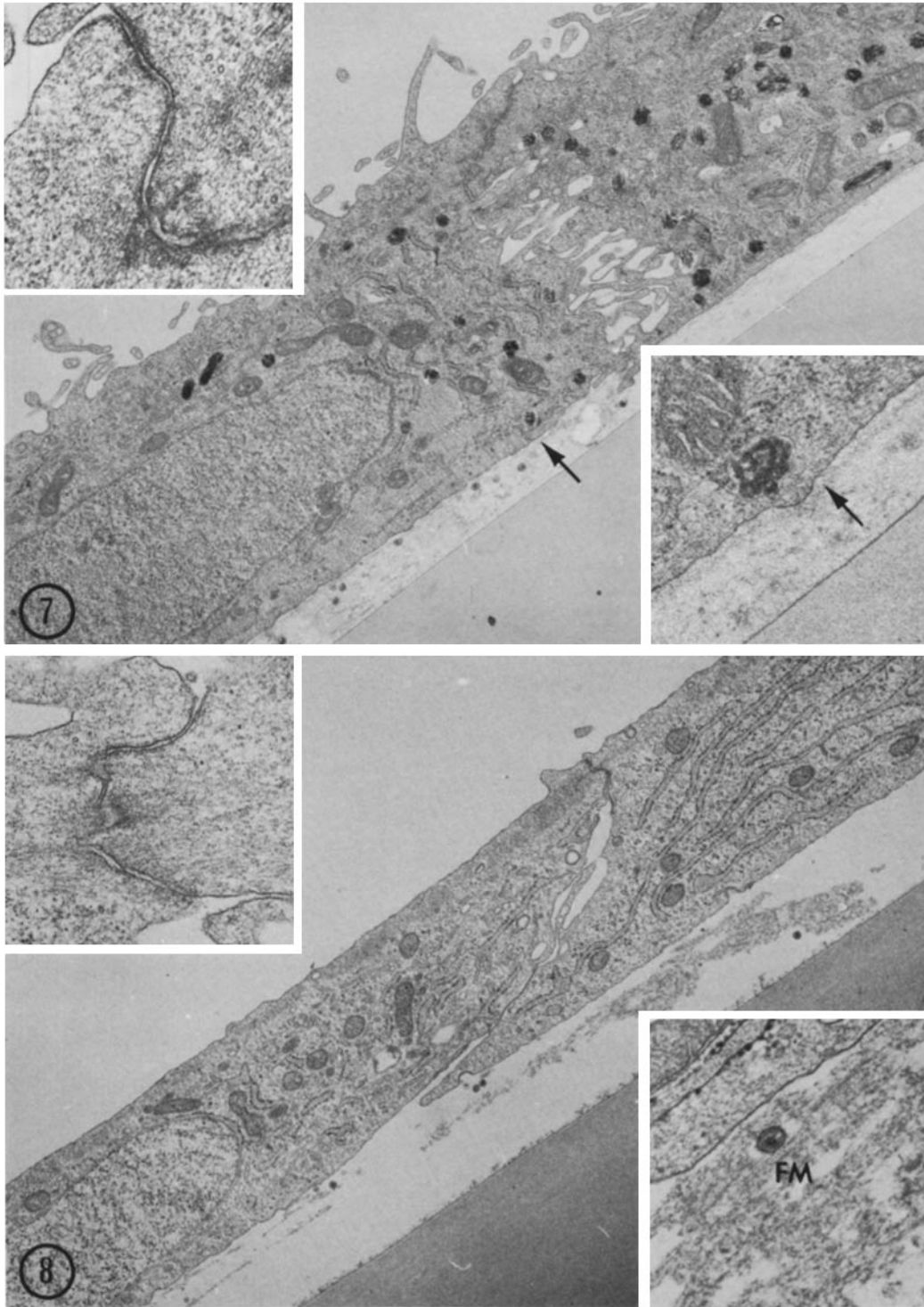
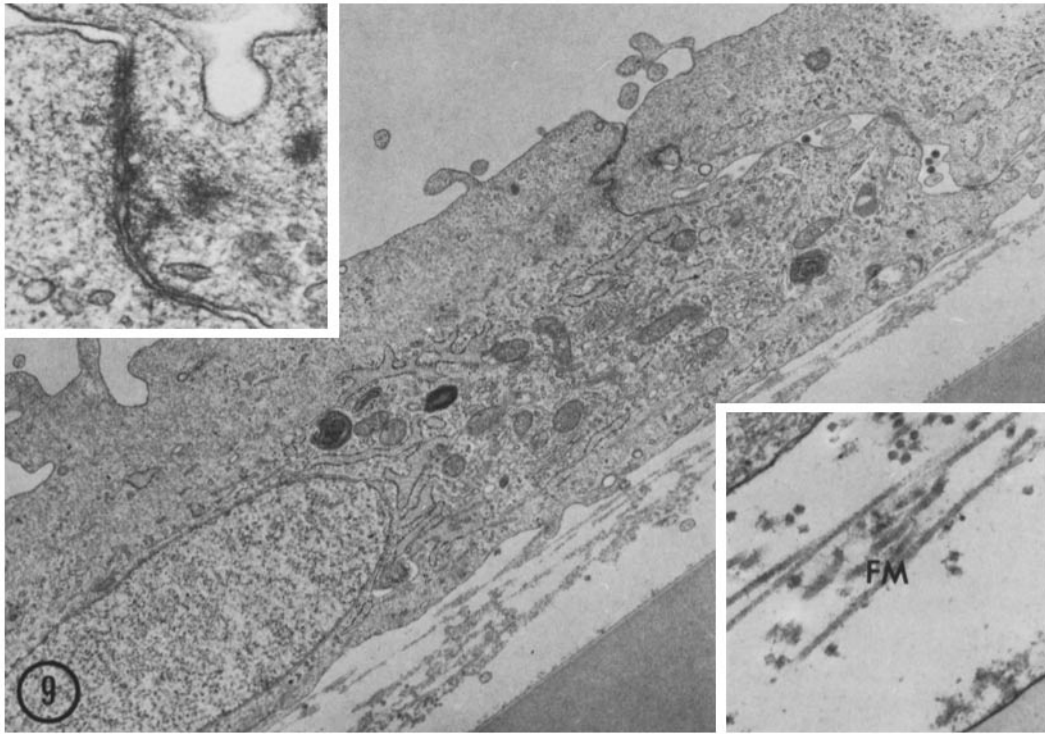
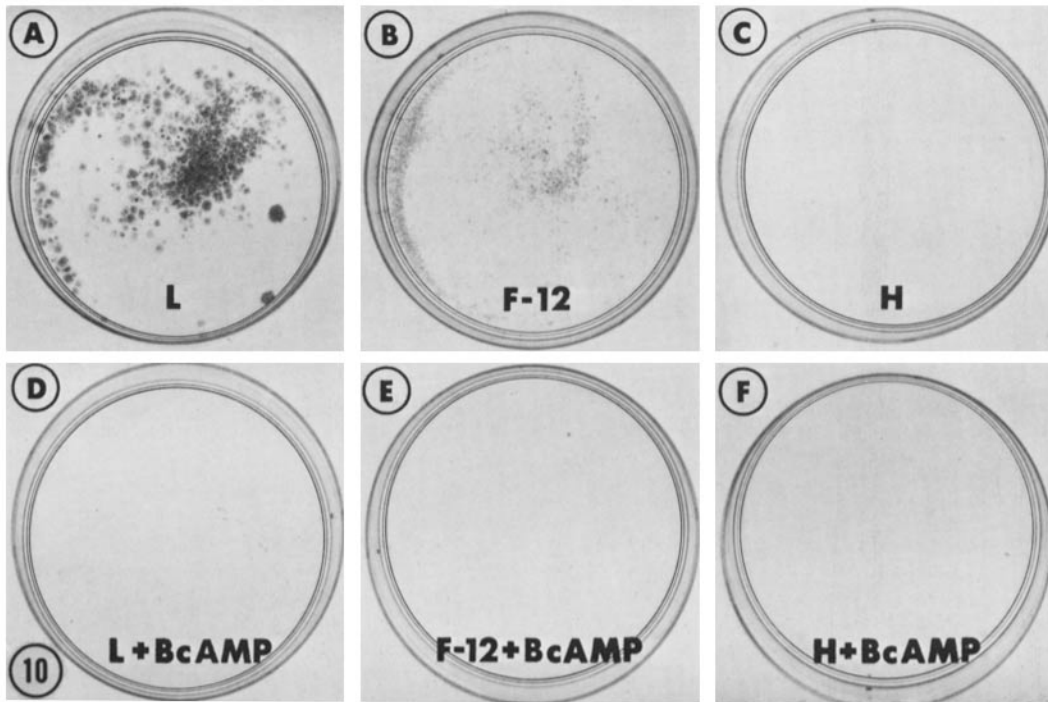


FIGURE 7 Transections of pigmented epithelial cells cloned from a stage 26 donor after 17 days of growth in L medium, showing a tortuous, but vertical cell boundary, basal lamina (arrow), and many pigment granules.  $\times 12,000$ . The upper *inset* shows an apical junctional complex, and the lower one demonstrates the basal lamina (arrow).  $\times 38,000$ .

FIGURE 8 Transections of cells from the same clone as those in Fig. 7, but grown for 17 days in H medium, showing an oblique, less tortuous cell boundary and a lack of basal lamina and pigment granules.  $\times 12,000$ . The upper *inset* shows an apical junctional complex and the lower one shows extracellular filamentous material (FM) in the basal region.  $\times 38,000$ .



**FIGURE 9** Transections of cells from the same clone as those in Figs. 7 and 8, but grown for 17 days in H medium with BcAMP ( $2 \times 10^{-4}$  M) added.  $\times 12,000$ . The upper *inset* shows an apical junctional complex, and the lower one shows some coarse filamentous material (FM).  $\times 38,000$ .



**FIGURE 10** Colonies of pigmented epithelial cells from the same clone as in Figs. 7-9 cultured for 17 days in Ham's F-12 medium with different modifications: (Fig. 10 A) addition of the low molecular weight fraction of chick embryo extract (L); (Fig. 10 B) no modification (F-12); (Fig. 10 C) addition of the heavy fraction of chick embryo extract (H); (Fig. 10 D) addition of L plus dibutyryl cyclic AMP (L + BcAMP); (Fig. 10 E) addition of BcAMP (F-12 + BcAMP); (Fig. 10 F) addition of H plus BcAMP (H + BcAMP).  $\times 0.7$ .

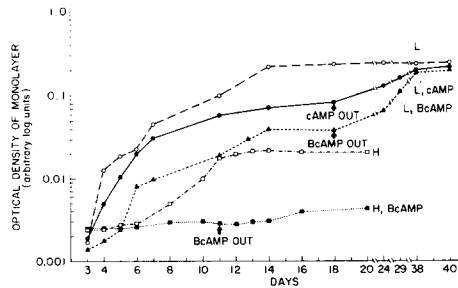


FIGURE 11 Effects of medium and BcAMP on visible pigmentation. Cells were grown in H medium ( $\square$ --- $\square$ ); H medium + BcAMP ( $\blacksquare$ ... $\blacksquare$ ); L medium ( $\circ$ --- $\circ$ ); L medium + cyclic AMP ( $\bullet$ — $\bullet$ ) or L medium + BcAMP ( $\triangle$ --- $\triangle$ ). Cyclic nucleotide was removed when indicated. Optical density was determined for four replicate samples at each time point; agreement was within 10%.

cells and induce pigment production. Wong and Pawalek (21) have also reported a mouse melanoma tumor cell line that responds either to melanocyte-stimulating hormone (MSH) or to cyclic AMP with melanin deposition and morphologic changes. In normal neural PE cells, we have been able to promote or retard visible pigmentation by the addition of the L or the H embryo fraction to the F-12 culture medium; cells grown in F-12 medium alone are intermediate in this regard. Our observation that BcAMP significantly retards visible pigmentation in PE cells grown in a medium that otherwise promotes differentiation was, therefore, quite unexpected. The present system is complex, however. There are most probably factors other than changes in cyclic AMP levels which influence the morphological and pigmentary characteristics of the cultured PE cells. It is possible, for example, that L medium contains a specific inducer (or H medium an inhibitor) of pigment production. Since the visible pigmentation of our cells increased after the cyclic nucleotide was removed from the medium, we feel justified in attributing this effect to cyclic AMP, but cannot yet say whether the effect on pigmentation is due to alterations in melanin synthesis, disposal, dispersion, or turnover rate.

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