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Further Changes in Differentiation State Accompanying the Conversion of Chinese Hamster Cells to Fibroblastic Form by Dibutyryl Adenosine Cyclic 3′:5′-Monophosphate and Hormones

(contact inhibition/collagen synthesis/agglutination)

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ABSTRACT **The morphological conversion *in vitro* of Chinese hamster ovary cells to a fibroblast form by a relatively large amount of dibutyryl adenosine cyclic 3′:5′-monophosphate, or by a combination of small amounts of this compound and testosterone, is attended by appearance of the following additional properties: acquisition of strict contact inhibition of growth; reorientation of the random growth pattern into one in which cells grow parallel to their long dimension; disappearance of the randomly distributed, knob-like, pseudopodal structures around the cell periphery; induction of collagen synthesis; and decrease in the ability to be agglutinated and rounded up by plant agglutinins and specific cell antibodies. The changes in these characteristics are consistent with the conversion from a malignant to a normal fibroblastic state. This conversion is under genetic control, as demonstrated by the production of specific mutants with altered characteristics. The response to testosterone is specific since steroids like estradiol and hydrocortisone are inactive, and others have limited activity. Some prostaglandins are equal in activity to testosterone and 5α-dihydrotestosterone. This system appears useful in study of the regulation of phenotypic expression in mammalian cells.**

In previous communications (1, 2) it was shown that treatment of Chinese hamster ovary cells *in vitro* with dibutyryl adenosine cyclic 3′:5′-monophosphate ($But_2A>p$) converts a compact, randomly oriented cell culture which grows in a multilayered form into a monolayer of elongated fibroblast-like cells which grow parallel to one another. Testosterone, which has a similar though smaller effect at high concentrations, potentiates the action of $But_2A>p$ so that maximal, morphological conversion can be obtained by the joint action of both agents in concentrations of each which are virtually inactive when used singly. The transformation affects cells throughout most of the life cycle, is completely reversible, need not materially change the rate of cell reproduction, and can be inhibited by agents, like colcemid and vinblastine, which prevent assembly of cellular microtubules. Similar findings concerning the action of $But_2A>p$, but not the hormone effect, have been reported by other investigators (3).

The change from contact-inhibited, monolayered growth to multiple-layered growth that does not exhibit contact inhibition, has been associated with the conversion of normal, differentiated cells to malignancy. Therefore, it was important to investigate aspects of the present reversible cell transformation that might illuminate the relevance of these observations to the problem of loss and gain of specific differentiation characteristics and their implication for cancer. The present paper describes further properties of this reversible system.

METHODS AND MATERIALS

The Chinese hamster ovary clone CHO-Kl, previously described, was used in all of these experiments (4). The properties of this cell line and procedures used in its cultivation have been reported (2, 4). The standard growth medium employed was F12, supplemented with the macromolecular fraction of fetal calf serum in a concentration equivalent to 10% of whole serum.

In studies on cellular agglutinability cells were grown in the presence or absence of $But_2A>p$ plus testosterone for 48 hr, and the quantitative agglutination assay was performed according to Pollack and Burger (5): Cells were harvested from tissue-culture flasks by ethylene dinitrilo tetraacetate and the resulting suspensions were washed and resuspended in isotonic saline to a concentration of 1–1.5 $\times 10^6$ cells per ml. Various concentrations of wheat germ agglutinin (WGA) were added and the mixture was allowed to stand at room temperature for 15 min, after which a drop was placed on a slide and examined under the microscope. The concentration of WGA (in μg/ml) that yields agglutination of 75% of the cells in randomly selected fields is taken as the end-point.

To study collagen synthesis, we grew cultures for 48 hr in 60-mm Petri dishes, at which point there are in each dish approximately 1–2 $\times 10^6$ cells in the late exponential phase of growth in each dish. After removal of the old medium, cultures were furnished with fresh medium containing 10^{-5} M L-proline, 1 μCi/ml of [^{3}H]L-proline (total, 5 μCi), and 50 μg/ml of sodium ascorbate, and incubated for 16 hr. Collagen synthesis was measured by the method of Juva and Prockop (6) as adapted by Priest and Davies (7). Total collagen synthesis was measured in the cells and in the medium.

Antisera to CHO cells were prepared and heated to destroy complement as described previously (8).

The various hormones and other agents used and their sources are as follows: $N^6,O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphoric acid (sodium salt), aldosterone (4-pregnen-11β,21-diol-3,18,20-trione), androstenedione (4-an-

Abbreviations: $But_2A>p$, $N^6,O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate; WGA, wheat germ agglutinin.

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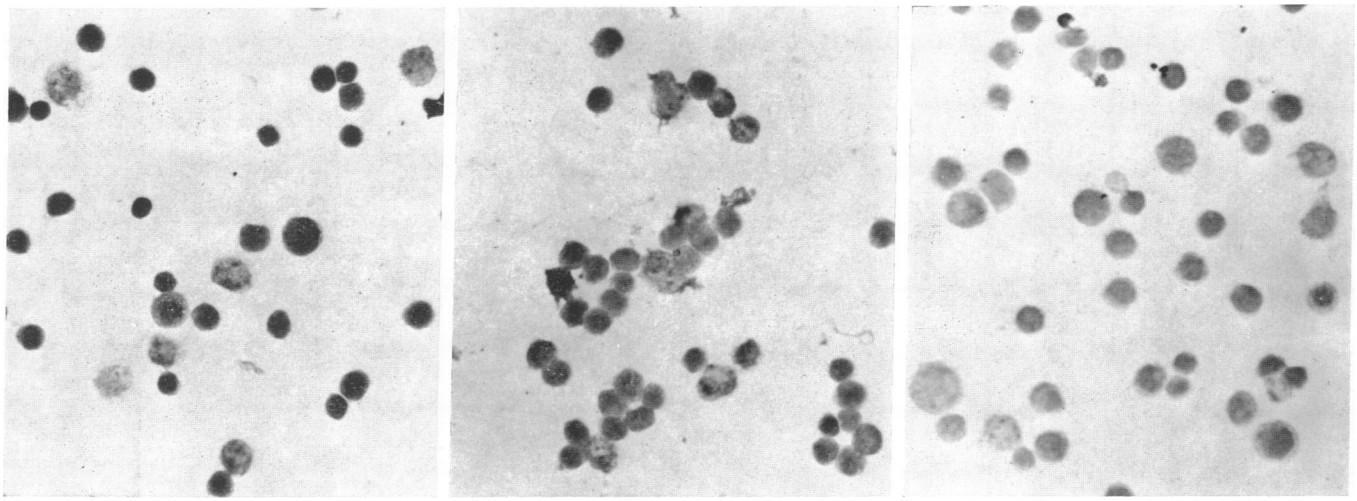

FIG. 1. Decrease in WGA agglutination produced by the addition of $But_2A>p$ and testosterone to growth medium. *Left*, cells grown in basal medium; no WGA. *Center*, cells grown in basal medium and tested with WGA, 30 μg/ml. *Right*, cells grown in basal medium plus 1 mM $But_2A>p$ or 0.2 mM $But_2A>p$ plus 15 μM testosterone, and tested with WGA, 30 μg/ml. $\times$150.

drosten-3,17-dione), cholesterol (5-cholesten-3β-ol), dehydroepiandrosterone (5-androsten-3β-ol-17-one), 11-deoxycorticosterone (4-pregnen-21-ol-3,20-dione), 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one), 17α-hydroxypregnenolone (5-pregnen-3β,17α-diol-20-one), 17α-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione), 11β-hydroxyprogesterone (4-pregnen-11β-ol-3,20-dione), 19-nor-testosterone (4-estren-17β-ol-3-one), and L-proline, Sigma Chemical Co.; 17β-estradiol [1,3,5(10)-estratrien-3,17β-diol 3-benzoate], pregnenolone (5-pregnen-3β-ol-20-one), testosterone (4-androsten-17β-ol-3-one proprionate), L-epinephrine, Mann Research Labs; hydrocortisone (4-pregnen-11,17,21-triol-3,20-dione sodium succinate), dexamethasone (1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3, 20-dione), prostaglandins A_1, A_2, E_1, E_2, and $F_{2\alpha}$ (kindly supplied to us by Dr. John E. Pike, The Upjohn Co.); progesterone (4-pregnen-3,20 dione), Brinkman Instruments, Inc.; concanavalin A, Calbiochem. Co. Glucagon-free insulin and insulin-free glucagon were gifts from Dr. J. M. McGuire, Eli Lilly and Co. Purified wheat germ agglutinin was generously supplied by Dr. John R. Sheppard, Department of Neurology, University of Colorado Medical Center, who prepared it according to Burger and Goldberg (9); [^{3}H]L-proline (740 Ci/mol) was from Amersham/Searle Co.

Aqueous stock solutions of 0.01 M $But_2A>p$ were made up and preserved in the freezer until use. All steroids (except hydrocortisone and dexamethasone in aqueous solutions) and prostaglandins were made up in 95–100% ethanol; epinephrine, glucagon, and insulin were dissolved in 0.01 N HCl at a concentration 100 times higher than the final concentration employed in the experiments. Solvent controls were constantly used to ensure that the solvent had no effect in the test system.

RESULTS

Action of cell-surface reagents

Aub, Tieslau, and Lankester (10) first demonstrated that an extract from wheat germ causes more marked agglutination of malignant than of normal cells. Subsequently, several plant glycoproteins have been found (11–13) to produce specific agglutination of spontaneously occurring cancer cells and cells transformed to a malignant form *in vitro* by physical, chemical or biological agents. In the present experiments to test cell agglutination, concanavalin A and WGA yielded similar results, although the former material is less active.

The agglutinability, by WGA, of the CHO cell was studied in its normal form and after its conversion by $But_2A>p$ plus testosterone to the fibroblast-like morphology. The results shown in Table 1 and Fig. 1 demonstrate that the conversion to the fibroblast-like, contact-inhibited form is attended by a clear decrease in agglutinability by WGA. The same effect is obtained regardless of whether the morphological change is brought about by 1 mM $But_2A>p$ or by 0.2 mM $But_2A>p$ plus 15 μM testosterone.

These agglutinating glycoproteins also induce a characteristic rounding reaction of the cells, forcing them to assume a spherical shape, even when grown upon a plastic surface. This reaction by concanavalin A is inhibited by the addition of $But_2A>p$ + testosterone.

Antibody preparations to CHO cells will attach to but not kill cells if complement is first eliminated by heating the antiserum (8). Like concanavalin A, such antisera can cause cells that have been grown attached to glass or plastic surfaces, so that they are flattened and spread out, to become almost spherical. This reaction is also prevented by $But_2A>p$ and testosterone. Fig. 2 illustrates the inhibition of this rounding reaction by $But_2A>p$.

These various experiments permit the conclusion that the reaction of $But_2A>p$ and testosterone with the cell involves a

TABLE 1. *Dibutyryl cyclic AMP and testosterone decrease the agglutinability of Chinese hamster ovary cells by wheat germ agglutinin*

Agents added	Concentration of WGA (μg/ml) necessary for end-point agglutination
None (control)	30
$But_2A>p$ (1 mM)	180
$But_2A>p$ (0.2 mM) + testosterone (15 μM)	180

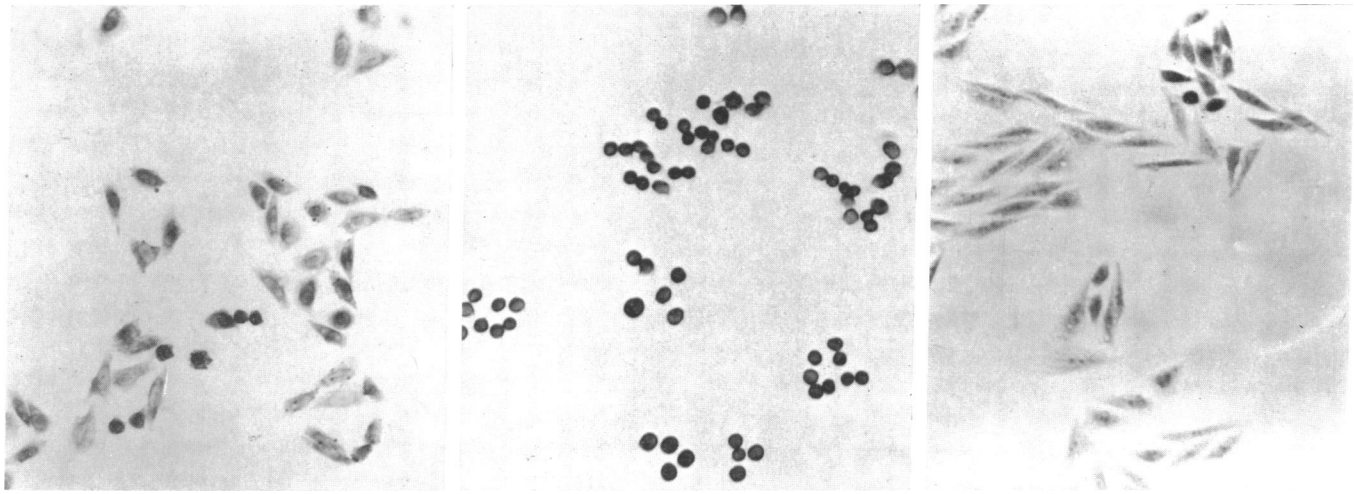

FIG. 2. Inhibition of the characteristic cell rounding reaction produced by concanavalin A by the presence of $But_2A>p$ or $But_2A>p$ plus testosterone in the medium. An almost identical set of plates was produced if antiserum to CHO-Kl cells was used instead of concanavalin A. *Left,* CHO cells grown in basal medium; *Center,* in basal medium plus 10 μg/ml of concanavalin A; *Right,* in basal medium plus 10 μg/ml of concanavalin A plus 1 mM $But_2A > p$. $\times 150$.

fundamental change in cellular surface elements. In addition they suggest that the biologically effective binding sites for concanavalin A are also antigenic sites on the cell surface.

Induction of collagen synthesis

Synthesis of collagen is a specific and characteristic property of the fibroblast. When cells are transformed to a malignant state, they frequently lose the ability to synthesize specific functional proteins characteristic of their original state of differentiation. Green, Todaro, and Goldberg (14) reported that virus-transformed 3T3 cells produce less collagen per unit of protein synthesis, under the specific conditions of their study, than do the parental fibroblast cells. We therefore determined the state of collagen synthesis in the epithelial-like and fibroblastic forms of the CHO cell. The data of Table 2 reveal a marked induction of collagen synthesis accompanying the change to the fibroblastic form.

Specificity of the testosterone reaction

It is important to determine whether the action of testosterone is a general one exhibited by all or many steroids, or whether its effectiveness in the reaction here studied is linked to its biologically specific activity. We tested several different steroids and other compounds in the standard basal medium plus 0.1 mM $But_2A>p$, which produces virtually no observable conversion to the fibroblastic morphology within 18 hr. However, when as little as 15 μM testosterone is present, maximal cell elongation is observed. The steroids named in Fig. 3 were tested in concentrations of 15 and 45 μM. The results demonstrate a high activity for the male steroid hormones, and no activity for the steroid estrogens. Other compounds like dehydroepiandrosterone, 17α-hydroxypregnenolone, and progesterone, which occur in regions of the biosynthetic chains common to both testosterone and 17β-estradiol, displayed various degrees of activity, but usually less than that of testosterone or 5α-dihydrotestosterone.

Other hormones known to exert cellular effects mediated through the cyclic AMP system were also tested. Such experiments show that insulin (0.017–17 μM), glucagon (0.028–28 μM), and epinephrine (1–100 μM) are ineffective. However, activity similar to that of testosterone was found in

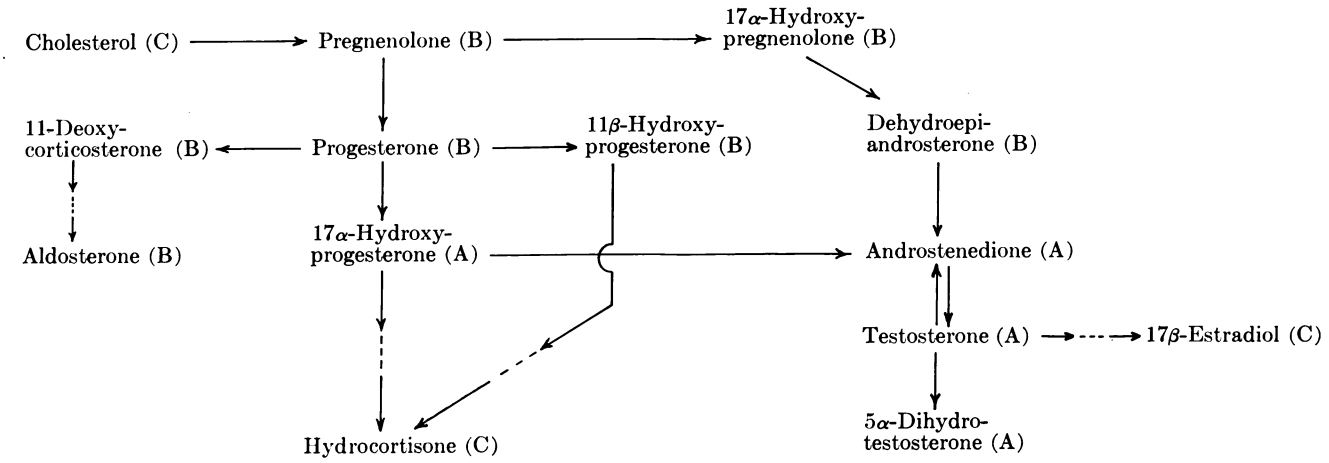

FIG. 3. Activity of various steroids in producing conversion of CHO-Kl cells to a fibroblast-like morphology when tested in the presence of 0.1 mM $But_2A>p$. *A*, high activity; *B*, some activity; *C*, no activity. The diagram is arranged to show the biosynthetic relationship of the steroids. Other steroids tested: 19-nortestosterone (*B*), dexamethasone (C).

TABLE 2. *Collagen synthesis induced by But₂A>p + testosterone in CHO cells*

Agents added	Cell morphology	[³H]Hydroxyproline protein (dpm/10⁶ cells)
None (control)	Epithelial	440
But₂A>p (1 mM)	Fibroblast-like	2680
But₂A>p (0.2 mM) + testosterone (15 μM)	Fibroblast-like	3920

Changes similar to those recorded in the last column of the table were noted in the ratio of newly synthesized collagen to newly synthesized total protein.

some of the prostaglandins, a family of lipid compounds with a wide variety of pharmacological activities. Prostaglandins E_1 and E_2 (15), each at a concentration of 57 μM, can cause significant cell elongation after incubation for 72 hr. Like testosterone, these lipids also exert synergistic effects with But₂A>p and in this action they are as potent as or even more potent than testosterone. Thus, in the presence of 0.1 mM But₂A>p, the action of 5.7×10^{-7} M prostaglandins E_1, E_2, A_1, or A_2 can be readily detected within 16 hr. Prostaglandin $F_{2\alpha}$ also has activity, but higher concentrations are required for its demonstration.

Other characteristics of the reaction

A particularly interesting feature of the morphological conversion involves the presence of knob-like structures with a high optical density extending from the cell periphery out into the external medium. In the epithelial form, these rounded, pseudopod-like extrusions are pronounced, expecially after about 18 hr of incubation, and 10 or more of such regions may be visible in the cell periphery (Fig. 4). When viewed by time-lapse cinemaphotomicrography, these regions are seen to be in active motion, extending into and retracting back from the outer environment. In contrast, the spindle-shaped fibroblastic form induced by the treatment has smooth edges, and no knob-like structures are visible, except occasionally at the two pointed tips. The optically dense regions in the knob-like structures might be sites of high mitochondrial concentration.

CHO cells can grow in suspension either in the presence or absence of But₂A>p plus testosterone, and in either case the cells assume a spherical shape. When these suspension-grown cells are plated in basal medium with or without But₂A>p and testosterone in plastic dishes and incubated in the humidified CO_2 incubator, the protruded knobs become visible within 10 min in either case. However, in the untreated cultures, the knobs persist for at least 24 hr, whereas in the cells treated with But₂A>p plus testosterone such knobs almost completely disappear within 1 hr as the cells assume an elongated form with a smooth periphery.

The potentiality for the interconversion of epithelial-like and fibroblastic forms is under genetic control, since mutants can be isolated with changed behavior in this respect. Mutants that display fibroblastic morphology even when grown on the basal medium alone have been isolated after treatment of CHO-K1 and other Chinese hamster cell cultures with ethylmethanesulfonate under conditions that produce high yields of single gene mutations (16). Properties of these mutants

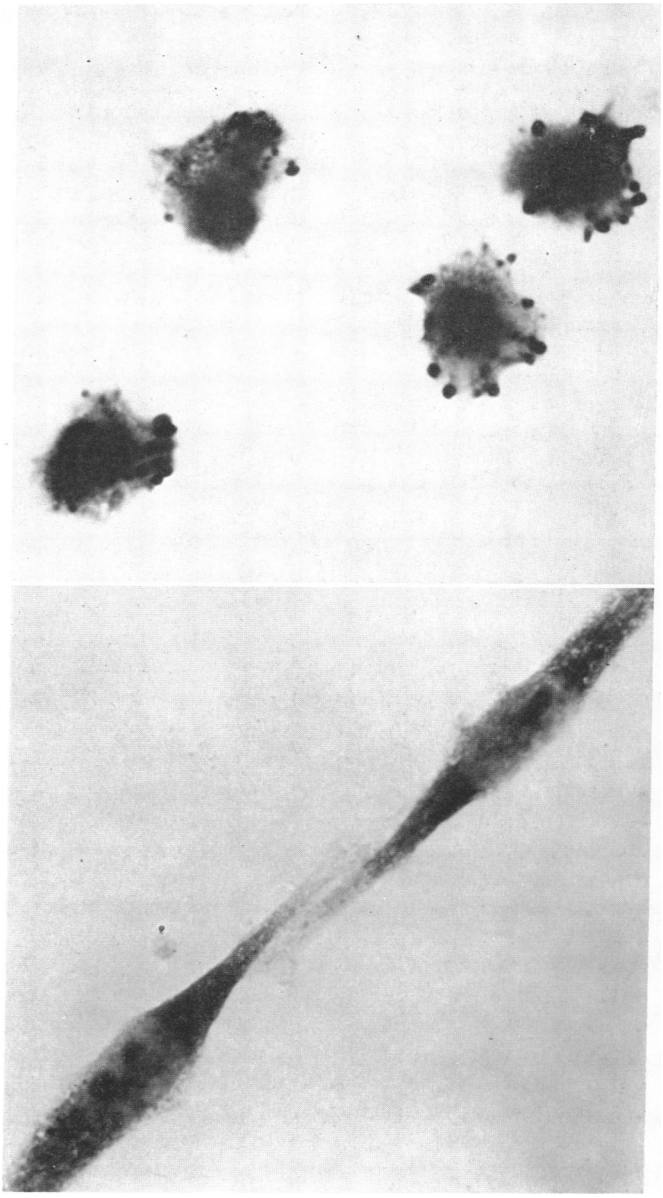

FIG. 4. Demonstration of the presence of knob-like, pseudopodal structures in CHO cells grown for 18 hr in basal medium (*above*), and the disappearance of these bodies in a similar culture to which But₂A>p and testosterone was added (*below*). ×1000.

will be described in detail in later publications, but in general they display rigorous contact inhibition as well as fibroblastic morphology, even in the basal medium. One of these, a mutant of an epithelial-like Chinese hamster lung cell, has also been shown to be less agglutinable by WGA, and to synthesize more collagen, than the parent cell from which it was derived. Availability of these mutants appears to furnish means for study of genetic aspects of this cellular modification.

DISCUSSION

In Table 3 are summarized all the changes in properties so far studied that accompany the conversion of the CHO cell to its fibroblast-like form by treatment with But₂A>p and testosterone under the prescribed conditions. Each of the processes listed in the table will be considered in detail in subsequent publications.

TABLE 3. *Properties of each form of the CHO cell*

Epithelial-like cell	Fibroblast-like cell
Compact cell morphology.	Spindle-shaped morphology.
Growth in multilayers.	Strict contact inhibition producing only monolayered growth.
Random cell orientation.	Cells grow in close association parallel to one another's long dimension.
Rough knob-like projections around the cell's periphery.	Smooth cell periphery, except for occasional knob-like structures at the pointed ends of the cell.
Little or no collagen synthesis.	Definite collagen synthesis.
Strongly agglutinated and rounded by WGA. Also rounded up by specific cell antibody.	Weakly agglutinated and rounded by WGA. Weakly rounded up by specific cell antibody.

The epithelial-like state of this cell is characterized by the ability to grow without contact inhibition, loss of the ability to synthesize a specific differentiation protein, namely collagen, and increase in the agglutinability by WGA—properties of mammalian cells that have been found to correlate with malignancy. Hence the properties of this system are of particular interest for the problem of cancer.

The fact that the reversible changes in these properties brought about by the agents studied here also occur as permanent changes after treatment with a mutagen is of special interest, and recalls the permanent changes associated with cell "transformation" to malignancy produced by carcinogens, many of which are demonstrated to be mutagens.

The mechanism of the transformation in properties here described is as yet obscure. The phenomena listed in Table 3 make it clear that a fundamental change in properties of the cell membrane has occurred. As a working hypothesis, we consider that this treatment may alter the permeability of the cell so that molecules concerned with covering or uncovering specific gene loci are more readily transferred across the altered cell membrane.

These experiments are particularly interesting because of the synergism they reveal between a cyclic AMP derivative and specific hormones like testosterone, 5α-dihydrotestosterone, and certain of the prostaglandins, but not other hormones like 17β-estradiol. Whether the synergistic action of testosterone occurs through enhancement of the transport of $But_2A>p$ into the cell, stimulation of the formation of cyclic AMP within the cell, or through some other means is under investigation. However, it is interesting in this connection that prostaglandin E_1, which exhibits an activity like that of testosterone, enhances A>p formation in several different types of cells (17). Comparison of the mechanism of action of the various agents involved in this transformation is obviously required.

The reactivity of this cell of ovarian origin to testosterone but not to estradiol is reminescent of the demonstration of sex specificity in binding sites for sex hormones on intracellular membranes of rat liver cells (18, 19). Activation of isolated endoplasmic reticulum from such cells required estradiol in the male, and testosterone in the female.

The present experiments indicate that a given induction of protein synthesis can be under the control of two separate systems, involving a steroid hormone on the one hand, and a A>p derivative on the other. Such an arrangement would appear appropriate for some kinds of hormonal regulation, since much more flexibility for control can be achieved with combinations of different agents than if each cellular response were mediated by a single substance.

The experiments reported here appear to be the first demonstration of the induction of synthesis of collagen, the most abundant protein of the mammalian body.

We are grateful to Dr. Robert E. Priest for assistance in development of the collagen assay employed and to Karen New for technical assistance. This investigation was aided by U.S. Public Health Service Grant No. 5 PO1 HD02080 from the National Institute of Child Health and Human Development and by American Cancer Society Grant No. E-642A. A. W. H. is a National Institutes of Health Postdoctoral Fellow, C. J. a Postdoctoral Trainee, USPHS Training Grant 5 TO1 GM00781, and T. T. P. an American Cancer Society Research Professor.

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