

# Inhibition of DNA Synthesis in Adrenocortical Cells by Cytochalasin B

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**ABSTRACT** ACTH inhibits DNA synthesis in normal rat and mouse tumor Y-1 adrenocortical cells within the same concentration range that it stimulates steroidogenesis. These processes can be independently regulated as demonstrated by the divergent actions of cytochalasin B on these cells.

In the normal cells, cytochalasin B does not increase steroidogenesis in serum-free or serum-containing media, and it decreases the stimulation produced by ACTH. In the absence of serum, the Y-1 cells respond in a similar way. However, in serum-containing media, cytochalasin B increases steroidogenesis in these cells and does not inhibit the response to ACTH.

In both cell types, cytochalasin B inhibits [<sup>3</sup>H]thymidine incorporation into DNA by a mechanism different than that of ACTH. In the Y-1 cells, this inhibition is caused by a decreased uptake of [<sup>3</sup>H]thymidine into the cell, which probably reflects a decreased transport across the cell membrane. In the normal cells, cytochalasin B, like ACTH, does not affect [<sup>3</sup>H]thymidine transport, but it decreases DNA synthesis much more rapidly than does ACTH. This inhibition may be the result of the disruption of microfilaments by cytochalasin B, because our evidence indicates that it is not caused by a decrease in glucose uptake by the cells.

ACTH plays a major role in regulating acute steroidogenesis and maintaining differentiated function of the adrenal gland. Although prolonged administration *in vivo* has been reported to increase adrenal DNA content (9, 11, 16), this may not be a direct effect of ACTH because other evidence (3, 4) indicates that ACTH causes hypertrophy rather than hyperplasia of the gland. We have recently shown (12) that in unilaterally adrenalectomized rats, administration of specific ACTH antiserum had no effect on the increase in DNA content and cell proliferation of the remaining adrenal gland. Furthermore, our studies *in vitro* have demonstrated that ACTH inhibits DNA synthesis and cell replication in normal rat adrenocortical cells (25) as it does in the mouse tumor Y-1 cells (17). We have now studied the regulation of steroidogenesis and DNA synthesis in both normal and tumor adrenocortical cells *in vitro*. To investigate whether these processes are necessarily linked, we have used cytochalasin B, an agent that disrupts microfilaments (32) and that has recently been reported to stimulate steroidogenesis in Y-1 cells in the presence of serum (6) but to inhibit the steroidogenic action of ACTH in the absence of serum (21). We report that the normal rat adrenocortical cells respond very differently to cytochalasin B than do the Y-1 cells, in terms of both steroidogenesis and DNA synthesis.

## MATERIALS AND METHODS

### Materials

Highly purified porcine ACTH was prepared as previously described (2). The cytochalasins, obtained from Sigma Chemical Co. (St. Louis, Mo.), were dissolved in 95% ethanol as stock solutions and then appropriately diluted into the incubation medium. In all cases, ethanol controls were run and the final concentration of ethanol was always lower than 0.25%. Sera, mycostatin suspension, and media were purchased from Grand Island Biological Co. (Grand Island, N.Y.). Gentamicin was purchased from Schering Corp. (Kenilworth, N.J.), collagenase (170 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, N.J.), and DNase (410 Kunitz U/mg) was obtained from Sigma Chemical Co. Tissue culture dishes were acquired from Falcon Plastics (Oxnard, Calif.). Radiochemicals were purchased from New England Nuclear (Boston, Mass.), and PCS liquid scintillation fluid was purchased from Amersham Corp. (Arlington Heights, Ill.). BSA (bovine serum albumin fraction V, purchased from Miles Laboratories, Inc. (Elkhart, Ind.)), was purified as previously described (27).

### Cell Culture

Normal adrenocortical cells were isolated from the adrenal glands of adult male Sprague-Dawley rats (140–180 g) by digestion with collagenase and DNase as previously described (25). The cells were suspended in medium 199 D-valine containing 10% dialyzed fetal calf serum, gentamicin (40 µg/ml) and Mycostatin (20 U/ml) and plated in 2-cm<sup>2</sup> multiwells at a density of 10<sup>5</sup> cells/ml per well. The results shown were obtained from experiments carried out 7 d after plating the cells. Similar results were obtained with cells 5 d after plating.

The mouse adrenal tumor cell line Y-1, introduced in culture by Buonassisi et al. (1), was obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown as monolayers in 75-cm<sup>2</sup> plastic tissue culture flasks (20 × 10<sup>6</sup> cells/flask) in Ham's F-10 medium containing 15% horse serum, 2.5% fetal calf serum, and 30 μg/ml gentamicin. Cells were subcultured once a week using trypsin (0.05% in normal saline, containing 0.02% EDTA). They were resuspended in medium and serum and plated 2-cm<sup>2</sup> multiwells at a density of 2 × 10<sup>5</sup> cells/ml per well. The results shown were obtained from experiments carried out 24 h after plating cells. Similar results were obtained with cells 5 d after plating.

All cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, and the medium was replaced every 2–3 d. Under these conditions, the Y-1 cells doubled every 24 h, whereas the normal cells had a doubling time of 48 h.

### Steroid Measurement

Cells were incubated in 1 ml of medium under the conditions stated in the figure legends. 50-μl aliquots of medium were removed at the end of the incubation and stored frozen at -20°C until assayed for steroid content. A specific radioimmunoassay for corticosterone described previously (12) was used to measure steroid output from the normal cells. The major steroid produced by the Y-1 cells is 20α-dihydroprogesterone (22, 15). This was also measured by a specific radioimmunoassay which gave a <0.01% cross-reactivity with cortisol, corticosterone, progesterone, and 17α-hydroxyprogesterone and a 0.03% cross-reactivity with testosterone.

### Measurement of [<sup>3</sup>H]Thymidine Incorporation

For these measurements the medium that was considered to be the most suitable for maintenance and growth of each cell type was used. Thus, the results shown were obtained using medium 199 D-valine with 10% dialyzed fetal calf serum (FCS) for the normal cells and Ham's F-10 with 15% horse serum (HS) and 2.5% FCS for the Y-1 cells.

Cells were incubated in 1 ml of medium in the presence or absence of hormones at 37°C. [<sup>3</sup>H]thymidine (5 Ci/mmol; 5 μCi/ml per well) was added and incubations were continued for the times stated in the figure legends. The reactions were terminated within 20 s by rapid washing of the cells with 1 ml ice-cold Dulbecco's phosphate-buffered saline (PBS) and immediate addition of 1 ml TCA (10% wt/vol) at 0°C and dissolved in 0.5 ml of 0.1 N NaOH by incubating at 37°C for 1–2 h. An aliquot was mixed with PCS scintillation fluid and counted in a Packard Tri-carb 3320 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

### Measurement of [<sup>3</sup>H]Thymidine Uptake

Cells were incubated at 37°C in 1 ml of medium (see above) containing [<sup>3</sup>H]thymidine, under the conditions stated in the figure legends. Reactions were terminated effectively within 20 s, by rapid washing of the cells with 1 ml of PBS at 0°C and immediate addition of 500 μl of sodium dodecyl sulfate (10% wt/vol). Total cell material was completely solubilized during a 1–2-h period at 37°C. The amount of radioactivity taken up was determined by counting an aliquot of the solubilized material.

## RESULTS

### Actions of Cytochalasin B

**STEROIDOGENESIS:** Cytochalasin B did not increase steroidogenesis in normal rat adrenocortical cells during a 1–24-h incubation. Fig. 1A shows the results of corticosterone output from cells incubated for 2 or 8 h with varying concentrations of cytochalasin B in the presence of serum. A similar lack of effect of cytochalasin B was observed in serum-free media such as medium 199 D-valine with 0.5% BSA or PBS with 0.5% BSA and in Ham's F-10 with 15% HS, 2.5% FCS.

In the Y-1 cells (Fig. 1B), cytochalasin B increased steroidogenesis two to threefold, in the presence of serum, over a 1–24-h incubation. The results of an 8-h incubation are shown in Fig. 1B.

**[<sup>3</sup>H]THYMIDINE INCORPORATION:** In both cell types, cytochalasin B decreased [<sup>3</sup>H]thymidine incorporation into TCA-insoluble material in a dose-dependent manner (Fig. 1). The normal cells were slightly less sensitive to inhibition than

the Y-1 cells, because a 1-μM concentration of cytochalasin B, which had no effect on the normal cells, decreased [<sup>3</sup>H]thymidine incorporation by ~30% after 24 h in the Y-1 cells (Figs. 1A and B).

### Time-course of Inhibition of [<sup>3</sup>H]Thymidine Incorporation by ACTH and Cytochalasin B

We have previously shown that ACTH decreases [<sup>3</sup>H]thymidine incorporation into DNA in normal rat adrenocortical cells in culture (25). Fig. 2 (A and B) shows that an inhibitory effect of ACTH was not seen until 4–6 h after incubation, in both the normal and the Y-1 cells. However, cytochalasin B inhibited [<sup>3</sup>H]thymidine incorporation by 40–50% in the normal cells and 25–35% in the Y-1 cells within the first 2 h of incubation, and a significant effect was observed as early as 30 min (Fig. 4C and D).

We have also investigated whether the characteristic morphological changes produced by both ACTH (33, 25) and cytochalasin B (6) in adrenocortical cells occur at the same time as inhibition of [<sup>3</sup>H]thymidine incorporation. In the Y-1 cells, ACTH and cytochalasin B cause very rapid morphological changes within 30 min of incubation (6). Similarly, in the normal rat adrenocortical cells, cytochalasin B caused cell rounding within 30 min, whereas an effect of ACTH was observed only after 4–5 h.

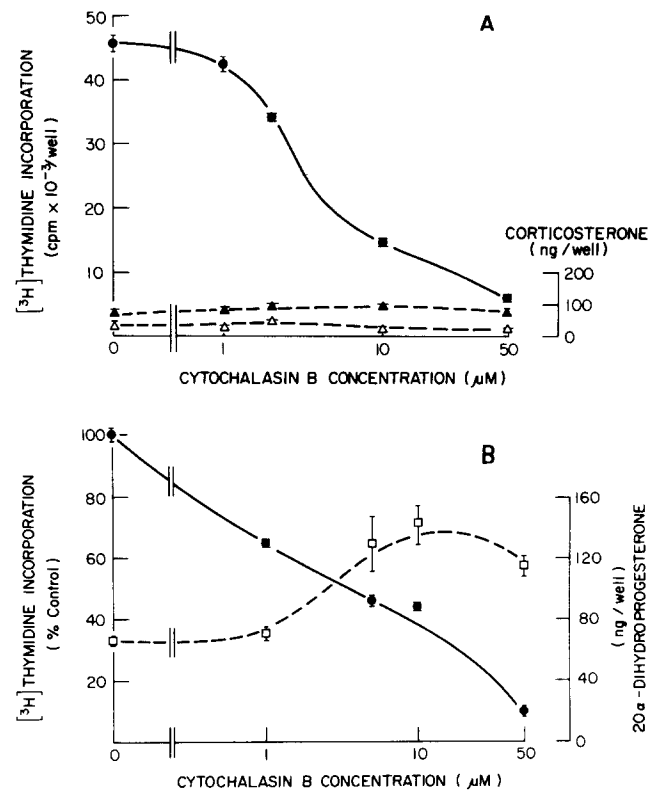


FIGURE 1 Actions of cytochalasin B on steroidogenesis and [<sup>3</sup>H]-thymidine incorporation in normal rat adrenocortical cells (A) or in mouse tumor Y-1 cells (B). Cells were incubated in 1 ml of medium in the presence of various concentrations of cytochalasin B. Aliquots of medium were taken after 2 h (Δ) and 8 h (▲ and □) for measurement of steroid production. [<sup>3</sup>H]thymidine (5 μCi/well; final concentrations, 0.5 μM [A] and 2.9 μM [B]) was added after 8 h, and the radioactivity incorporated into TCA-insoluble material during a subsequent 40-h (A) or 16-h (B) incubation was determined (●). Results are means ± SEM for six (A) and four (B) determinations.

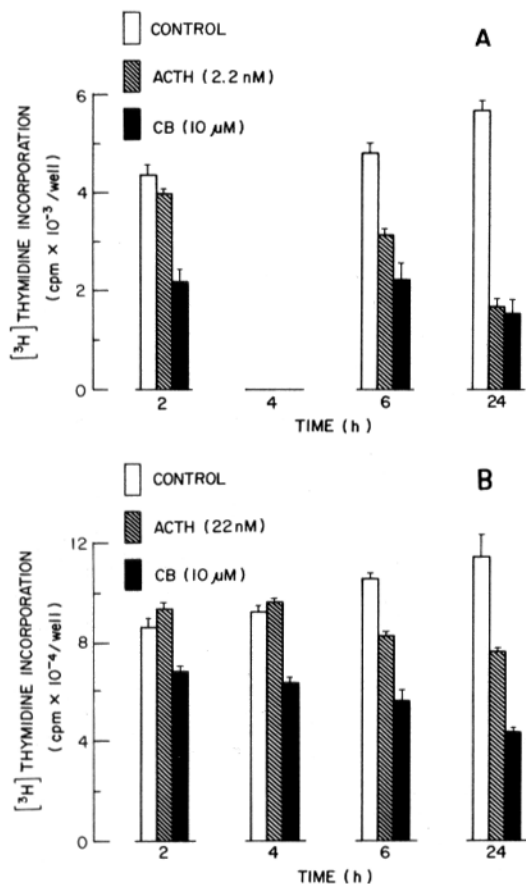


FIGURE 2 Time-course of inhibition of  $[^3\text{H}]$ thymidine incorporation by ACTH and cytochalasin B in normal cells (A) or Y-1 cells (B). Cells were incubated in 1 ml of medium in the presence or absence of ACTH or cytochalasin B for the times shown. They were pulsed with  $[^3\text{H}]$ thymidine (5  $\mu\text{Ci}$  per well; final concentrations, 0.1  $\mu\text{M}$  [A] and 2.9  $\mu\text{M}$  [B]) for the final 2 h (A) or 30 min (B) of incubation, and the TCA-insoluble radioactivity was measured. Results are means. Vertical bars extend to the limits of the range for three determinations.

### Divergent Effects of Cytochalasin B on ACTH-stimulated Steroidogenesis

In the normal rat adrenocortical cells incubated in the presence of serum (Fig. 3), cytochalasin B partially inhibited ACTH-stimulated steroidogenesis. This inhibitory effect was also seen in various serum-free media (Ham's F-10 with 0.5% BSA; medium 199 with 0.5% BSA; PBS with 0.5% BSA).

In the Y-1 cells, cytochalasin B had no effect on ACTH-stimulated steroidogenesis in serum-containing media (Ham's F-10 with 15% HS, 2.5% FCS or medium 199 D-valine with 10% dialyzed FCS), although it inhibited the stimulation by ACTH in serum-free media (data not shown).

### Effects of Cytochalasin B on ACTH Inhibition of $[^3\text{H}]$ Thymidine Incorporation

ACTH decreased  $[^3\text{H}]$ thymidine incorporation into TCA-insoluble material in a dose-dependent manner, in both cell types. The normal rat adrenocortical cells were very sensitive to inhibition by ACTH; physiological concentrations that increased steroidogenesis also decreased  $[^3\text{H}]$ thymidine incorporation. The  $K_m$  for ACTH for affecting both of these processes

in the normal cells was 15–30 pM, whereas in the Y-1 cells it was 250–500 pM.

When ACTH and cytochalasin B were added to the cells at the same time, the highest concentrations of ACTH were able to increase the maximum inhibition produced by cytochalasin B alone, in both cell types, although the combined effects were not additive (data not shown).

### Actions of Cytochalasin B on Thymidine Uptake and DNA Synthesis

Because cytochalasin B inhibited  $[^3\text{H}]$ thymidine incorporation into DNA very rapidly in both cell types, we investigated the possibility that this effect may be mediated by inhibition of  $[^3\text{H}]$ thymidine uptake into the cell. Results of experiments measuring uptake of  $[^3\text{H}]$ thymidine into total cell material are shown in Fig. 4A and B. The kinetics of uptake are very different; in the normal cells (Fig. 4A) the radioactive pool was saturated within 10 min, whereas in the Y-1 cells (Fig. 4B) there was a continual increase in uptake into the cell, over a 2-h period.

It can be seen that in the normal cells (Fig. 4A) no inhibitory effect of cytochalasin B on  $[^3\text{H}]$ thymidine uptake was observed. However, in the Y-1 cells (Fig. 4B), cytochalasin B inhibited  $[^3\text{H}]$ thymidine uptake into total cell material very rapidly, an effect being observed as early as 2 min. This decreased uptake could explain the rapid inhibition of  $[^3\text{H}]$ thymidine incorporation into DNA by cytochalasin B in these cells (Fig. 4C and D).

Similar results were obtained with Y-1 cells that had been maintained in low serum (0.2% FCS) medium containing 0.5% BSA for 48 h (data not presented). These cells incorporated less thymidine and had a growth rate which more closely approached that of the normal cells. The thymidine uptake curve obtained showed saturation kinetics, as in the case of the normal cells (Fig. 4, A). In these Y-1 cells, also, the inhibitory effects of cytochalasin B on thymidine incorporation could be explained by a decreased uptake of thymidine into the cell.

In both cell types, ACTH did not inhibit  $[^3\text{H}]$ thymidine uptake. In the normal cells, control uptake was  $1,884 \pm 151$  cpm (nine observations); in the ACTH (2.2 nM)-treated cells the uptake was  $1,819 \pm 107$  cpm (six observations) after a 2-min incubation. In the Y-1 cells, control uptake was  $14,649 \pm 487$  cpm (five observations); in the ACTH (22 nM)-treated

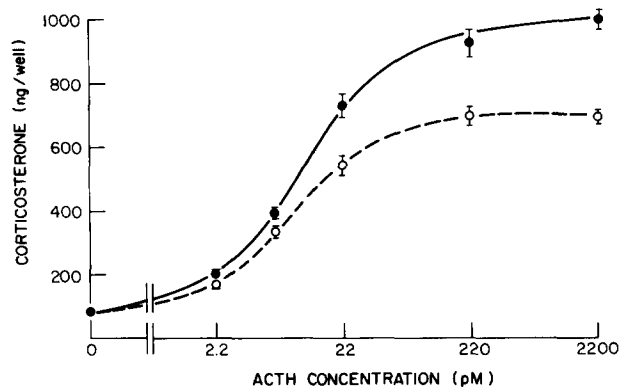


FIGURE 3 Effects of cytochalasin B on ACTH stimulation of steroidogenesis in normal cells. Cells were incubated with various concentrations of ACTH in the presence (○) or absence (●) of cytochalasin B (10  $\mu\text{M}$ ). Steroid output was measured after 8 h. Results are means  $\pm$  SEM for four determinations.

cells the uptake was  $13,751 \pm 781$  cpm (five observations) after a 2-min incubation. The differences between control and ACTH-treated cells were not significant.

The results of experiments in which the cells were prelabeled with [ $^3$ H]thymidine on ice for 30 min and then incubated at 37°C in the presence or absence of cytochalasin B are shown in Fig. 4, E and F. These experiments are designed to bypass possible effects on uptake into the cell because at 4°C, the

intracellular thymidine pool is labeled but no incorporation of thymidine into DNA occurs. On subsequent incubation of the cells at 37°C, incorporation of this intracellular labeled thymidine into DNA occurs. Hence, inclusion of cytochalasin B at this stage will show whether it is directly affecting DNA synthesis. In the normal cells (Fig. 4E) after prelabeling, cytochalasin B decreased [ $^3$ H]thymidine incorporation into TCA-insoluble material during a subsequent 30-min incuba-

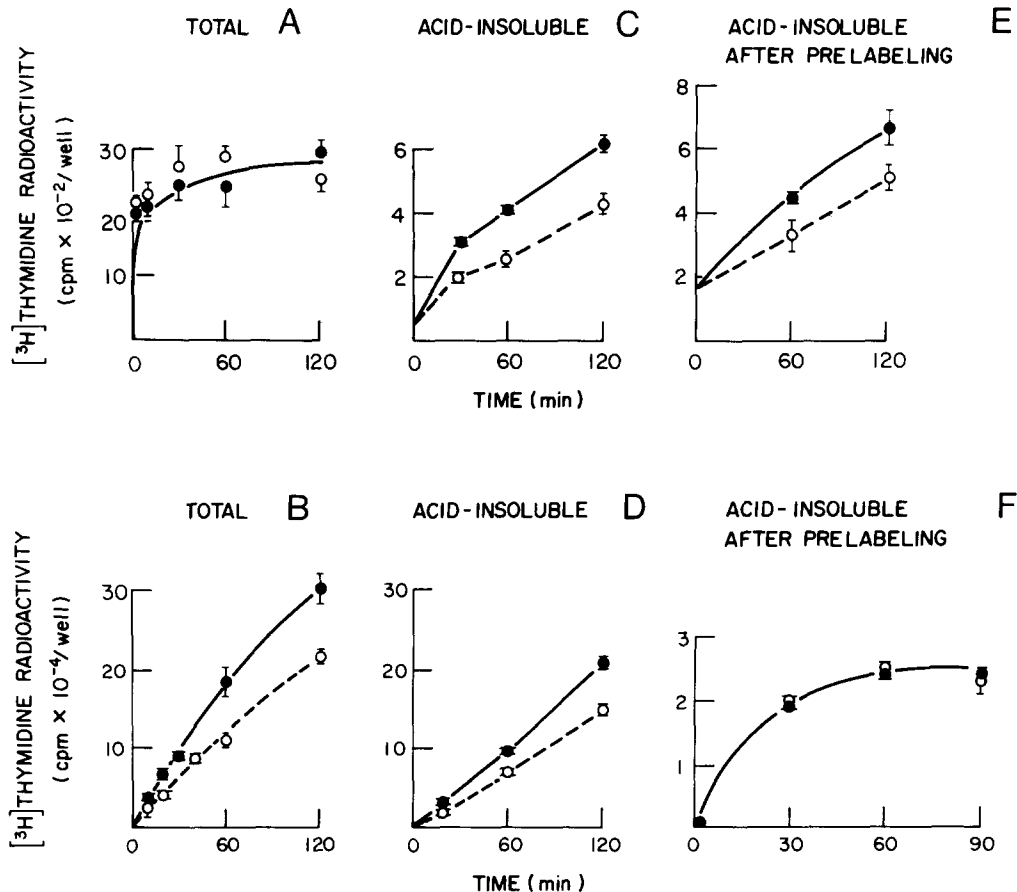


FIGURE 4 Actions of cytochalasin B on thymidine uptake and DNA synthesis in normal (A) and Y-1 (B) cells. (A-D) Cells were incubated in control medium (●) or medium containing 10  $\mu$ M cytochalasin B (○) for the times shown. [ $^3$ H]thymidine (5  $\mu$ Ci/well; final concentration, 0.1  $\mu$ M [A] and 2.9  $\mu$ M [B]) was added at zero time. A and B show results of [ $^3$ H]thymidine uptake into total cell material. C and D show results of [ $^3$ H]thymidine incorporated into TCA-insoluble material. (E and F) Cells were pre-labeled on ice in 1 ml of Dulbecco's modified Eagle medium (DME) with 15% dialyzed FCS that contained [ $^3$ H]thymidine (25  $\mu$ Ci/well; final concentration, 0.5  $\mu$ M [A]; 5  $\mu$ Ci per well; final concentration, 0.1  $\mu$ M [B] for 30 min). The cells were washed once with ice-cold DME with 15% dialyzed FCS and then incubated in 1 ml of this medium at 37°C in the presence (○) or absence (●) of cytochalasin B (10  $\mu$ M). [ $^3$ H]thymidine incorporated into TCA-insoluble material was determined as described in Materials and Methods.

TABLE I  
Effects of Cytochalasin D on [ $^3$ H]Thymidine Incorporation in Normal Rat Adrenocortical Cells

		[ $^3$ H]Thymidine incorporation, CPM/well			
Incubation time	Concentration of cytochalasin, $\mu$ M	0	2	5	10
8 h	B		9,887 $\pm$ 400	7,175 $\pm$ 390	6,050 $\pm$ 334
	D	11,762 $\pm$ 306	7,585 $\pm$ 480	7,173 $\pm$ 195	6,235 $\pm$ 218
24 h	B		11,422 $\pm$ 264	6,847 $\pm$ 555	4,095 $\pm$ 375
	D	17,184 $\pm$ 77	6,782 $\pm$ 309	5,964 $\pm$ 241	4,880 $\pm$ 202

Cells were incubated in medium containing varying concentrations of either cytochalasin B or cytochalasin D, for the times shown. For the final 2 h of incubation, the cells were pulsed with [ $^3$ H]thymidine (5  $\mu$ Ci per well, final concentration, 0.1  $\mu$ M) and the radioactivity incorporated into TCA-insoluble material was determined. Results are means  $\pm$  SEM for three determinations.

tion, suggesting that DNA synthesis was inhibited. Furthermore, 7 d after plating, the normal cells were treated with control or cytochalasin B (10  $\mu$ M)-containing medium for 48 h, and the DNA content of the cells was measured by the method of Ceriotti (5, 28). Control cells contained  $1.12 \pm 0.16 \mu$ g DNA, (3) whereas the cytochalasin B-treated cells contained  $0.52 \pm 0.04 \mu$ g DNA (3). Conversely, in the Y-1 cells no effect of cytochalasin B was observed after prelabeling, suggesting that DNA synthesis was not inhibited.

Representative experiments measuring [ $^3$ H]thymidine incorporation and uptake as described above were carried out with medium 199 D-valine with 10% dialyzed FCS for the Y-1 cells and Ham's F-10 with 15% HS with 2.5% FCS for the normal cells. The effects of cytochalasin B on the two cell types were similar to those already presented (data not shown). Thus, the marked differences observed in the responses of the two cell types to cytochalasin B were not caused by differences in medium composition or thymidine concentration.

### Effects of Cytochalasin D

Cytochalasin D, which is much less potent in inhibiting glucose uptake in various cell types (30) than is cytochalasin B, also inhibited [ $^3$ H]thymidine incorporation into DNA in the normal cells (Table I). At lower concentrations, it was more effective than cytochalasin B, although the same maximum effect was produced, suggesting a similar mechanism of action. Cytochalasin D caused morphological changes similar to those produced by cytochalasin B within a 30-min incubation.

### DISCUSSION

The mouse adrenal tumor cell line Y-1 has been used extensively as a model system to study the mechanisms by which steroidogenesis and DNA synthesis are regulated (17, 25, 6, 31). However, in the present paper we have shown that the Y-1 cells are not only much less sensitive to ACTH, but also respond very differently to cytochalasin B than do normal rat adrenocortical cells, in culture.

In the normal cells, cytochalasin B did not increase steroidogenesis in the presence or absence of serum and it inhibited the stimulation by ACTH. In the Y-1 cells, we confirm the results of previous studies (21, 6) showing that ACTH-stimulated steroidogenesis was inhibited only in serum-free media, and, in the presence of serum, cytochalasin B increased steroidogenesis. Thus, in the absence of serum, the Y-1 cells respond in the same way as the normal cells. The mechanism by which cytochalasin B increases steroid output in the Y-1 cells is different from that of ACTH, because ACTH stimulation is independent of the serum content of the medium and the maximum effect is greater than that produced by cytochalasin B.

In the normal rat adrenocortical cells, ACTH increases steroidogenesis and decreases [ $^3$ H]thymidine incorporation into DNA over the same physiological concentration range. It is apparent that these processes can be independently regulated, because cytochalasin B decreased [ $^3$ H]thymidine incorporation into DNA without increasing steroidogenesis and inhibited ACTH-stimulated steroidogenesis while increasing its inhibitory effect on [ $^3$ H]thymidine incorporation. The inhibitory effects of both ACTH and cytochalasin B on [ $^3$ H]thymidine incorporation into the normal cells are the result of decreased DNA synthesis, since we have shown that neither ACTH nor cytochalasin B has any effect on [ $^3$ H]thymidine uptake into the cell. This would be expected from our previous results (25, 26)

showing that ACTH-treated cultures have a lower DNA content and cell number than controls and from our present data showing that the cytochalasin B-treated cultures also contain less DNA than control.

Unlike the effect of ACTH, the inhibition by cytochalasin B of thymidine incorporation into the Y-1 cells can be explained by a decreased uptake of [ $^3$ H]thymidine into the cell. This is consistent with the rapid effect of cytochalasin B and the fact that ACTH and cytochalasin B together cause a greater effect than the maximum inhibition produced by either agent alone.

It is known (24) that transport of nucleotides and transport of nucleosides have very different characteristics, depending on the cell type. Thus, cytochalasin B inhibits thymidine transport in Chinese hamster ovary cells (8) and Novikoff rat hepatoma cells (23) but not in HeLa cells (20). In the Y-1 cells, it seems likely that cytochalasin B is inhibiting carrier-mediated transport, whereas it is possible that thymidine is entering the normal cells by simple diffusion or is being transported by a carrier system which is insensitive to inhibition by cytochalasin B.

Because cytochalasin B inhibits glucose uptake in a wide variety of cells (24, 20), we have investigated whether its rapid inhibitory effect on DNA synthesis in the normal cells is mediated by a decrease in glucose uptake. There is some dispute as to whether inhibition of glucose transport by cytochalasin B is competitive (7, 14) or noncompetitive (20, 34); however, in physiological media there is probably sufficient glucose available from endogenous stores or by diffusion such that overall glucose metabolism is not significantly altered. In the Y-1 cells (21) and in pituitary tissue (18), cytochalasin B did not affect ATP concentration during a 2-h incubation. Furthermore, increasing the glucose concentration of the incubation medium of the normal adrenocortical cells from 5.5 to 30 mM did not overcome the inhibitory effect of cytochalasin B on [ $^3$ H]thymidine incorporation (data not included).

We have also shown that cytochalasin D is more effective at lower concentrations than cytochalasin B in decreasing DNA synthesis in the normal cells. This analogue is much less potent in inhibiting glucose uptake than is cytochalasin B and is totally ineffective in some systems (19, 30). However, it is more effective in disrupting microfilaments and causes morphological changes similar to those produced by cytochalasin B in adrenocortical cells. Thus, the inhibition of DNA synthesis by cytochalasin B in the normal cells does not appear to be mediated by effects on glucose transport.

The inhibitory actions of both ACTH and cytochalasin B on DNA synthesis in these cells may be mediated by disruption or rearrangement of microfilaments since in both cases morphological changes are observed at the onset of inhibition of DNA synthesis. ACTH decreases DNA synthesis in both normal and tumor adrenocortical cells, whereas cytochalasin B inhibits only in the normal cells. The reason for this is not clear, but it may be related to the fact that cytochalasin B inhibits nuclear division in normal cells (13, 10, 29) but has no effect on a variety of tumor cell types.

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