

# Glucocorticoid Effects on HeLa S<sub>3</sub> Cell Growth and Thymidine Incorporation<sup>1</sup>

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## ABSTRACT

Growth of HeLa S<sub>3</sub> cells in monolayer cultures of Joklik's minimum essential medium, 5% fetal calf serum, and 2 mM glutamine in the presence of 10<sup>-6</sup> M dexamethasone results in an ~70% inhibition of exogenously added [<sup>3</sup>H]thymidine incorporation into DNA. In marked contrast, dexamethasone does not alter HeLa S<sub>3</sub> cell growth rate under these conditions. The half-maximal inhibitory concentration of dexamethasone is ~10<sup>-9</sup> M which correlates well with the dissociation constant of the nuclear glucocorticoid receptor at 37°. Only active glucocorticoids, e.g., dexamethasone and cortisol, inhibit [<sup>3</sup>H]thymidine incorporation into DNA. 17β-Estradiol, 5α-dihydrotestosterone, progesterone, and cortisone are ineffective. A measurable effect of dexamethasone at 10<sup>-8</sup> M occurs within 3 to 4 hr after hormone administration. The presence of transcriptional and translational inhibitors during exposure of the HeLa S<sub>3</sub> cells to glucocorticoids blocks the accumulation of the hormone effect. Dexamethasone has little or no effect on uridine and leucine incorporation into RNA and protein, respectively, under these conditions. These results demonstrate that the incorporation of a DNA precursor is regulated by glucocorticoid hormones in HeLa S<sub>3</sub> cells. This effect is most likely mediated via an alteration in the thymidine precursor pool specific activity.

## INTRODUCTION

Supplementation of tissue culture medium with radiolabeled thymidine is a commonly used method for the assessment of cell growth rate and DNA synthetic rates (4). Although exogenous thymidine is not an essential factor for cell growth in most tissue culture cells, the prevalence of thymidine kinase salvage pathways enables exogenous thymidine to be transformed to thymidine triphosphate for use in DNA synthesis. The use of exogenously supplied [<sup>3</sup>H]thymidine to measure DNA synthesis is complicated by at least 3 factors. (a) Detailed studies by Skoog *et al.* (15) and Walters *et al.* (18) indicate that endogenously synthesized pools of thymidine triphosphate and exogenously supplied radiolabeled pools of [<sup>3</sup>H]thymidine triphosphate differ significantly in their respective rates of intracellular degradation. (b) The metabolism of [<sup>3</sup>H]thymidine has been shown recently to be a cell cycle stage-dependent process (17), and (c) Thymidine uptake in cell cultures is a carrier-mediated process (9-12, 16) which, like that for sugars and amino acids, may be regulated by environmental factors present in tissue culture medium (6).

Our interest in the use of [<sup>3</sup>H]thymidine as a measure of DNA synthesis and cell growth stemmed from our attempts to rein-

vestigate the effects of glucocorticoids on HeLa S<sub>3</sub> growth parameters. Previous studies by Kollmorgan and Griffin (5) have demonstrated that high concentrations of cortisol, ~2 × 10<sup>-6</sup> M, did not alter cell-doubling time after a short lag period of 10 hr. Our results (2) support these earlier studies, although the growth lag described by Kollmorgan and Griffin (5) following glucocorticoid supplementation of tissue culture medium appeared to be more variable in our culture system. Routinely, during the course of our investigations, we observed that treatment of HeLa S<sub>3</sub> cells with dexamethasone, as well as natural glucocorticoids, markedly suppressed [<sup>3</sup>H]thymidine incorporation into DNA.

## MATERIALS AND METHODS

HeLa S<sub>3</sub> cells were grown in plastic tissue culture flasks and in glass spinner bottles in a 37° incubator equilibrated with 5% CO<sub>2</sub>:95% humidified air atmosphere. The cells were grown in Joklik's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum, 2 mM glutamine, penicillin G (75 units/ml), and streptomycin sulfate (50 units/ml). Steroids purchased from Steraloids (Wilton, N. H.) were prepared in stock solutions of ~2 × 10<sup>-4</sup> M in absolute ethanol and stored at -20° prior to use. For addition to cells, aliquots of each steroid solution were placed in glass tubes, the ethanol was evaporated, and the steroids were redissolved in cell growth medium. The concentrations shown in each chart or legend are the final concentrations achieved.

In experiments involving HeLa S<sub>3</sub> cells grown in monolayer conditions, cell harvesting was accomplished by replacing the growth medium with a solution of Versene (Grand Island Biological Co.) for ~15 min at 37° and collecting the cells by centrifugation at 500 × g for 5 min. Cell number was determined in a Model Zf Coulter Counter (Coulter Electronics, Hialeah, Fla.), and cell viability was assessed in a hemacytometer using the exclusion of trypan blue dye as a criterion for viability. Cell viability was consistently greater than 95%. Radioactivity was determined in a Beckman LS-150 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.) having an efficiency of 43% for tritium. Samples were counted in minivials containing 3.0 ml of a 2:1 v/v mixture of PCS (Amersham/Searle Corp., Arlington Heights, Ill.):xylene (Fisher, Scientific Co.).

Thymidine incorporation studies utilized [*methyl*-<sup>3</sup>H]thymidine (46 Ci/mmol) purchased from Amersham/Searle Corp. In most experiments, HeLa S<sub>3</sub> cells grown in 24-well cluster plates (Costar Scientific Products) were pulsed with trace quantities (2 μCi/ml) of [<sup>3</sup>H]thymidine prepared in growth medium. The incorporation reactions were terminated by cooling the cluster plates to 0° on ice and removing the radioactive growth medium. The cells were subsequently removed from each well

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with Versene, the wells were washed with ~700  $\mu$ l of 0.85% NaCl, and the cell pellets were collected by centrifugation at 1500  $\times$  *g* for 10 min in a Beckman TJ6 centrifuge maintained at 4°. The supernatants associated with cell pellets were discarded, and 2.0 ml of ice-cold 10% TCA<sup>3</sup> were added to each group of cells. The TCA precipitates were then centrifuged for 20 min at 1500  $\times$  *g* at 4°, and the supernatant was discarded. The TCA precipitates were then washed with an additional 2.0 ml 10% ice-cold TCA and recentrifuged in order to remove free acid-soluble [<sup>3</sup>H]thymidine or metabolites. The tips of each plastic tube containing the radioactive TCA precipitates were cut off with a razor blade and placed into scintillation vials. Three ml of scintillation fluid were added to each, and the samples were vortexed vigorously for 1 to 2 min to break up and solubilize the pellets.

## RESULTS

Chart 1 shows the results of an experiment in which HeLa S<sub>3</sub> cells were plated in 24-well cluster plates in Joklik's medium containing 5% fetal calf serum in the presence or absence of various concentrations of dexamethasone. On daily intervals thereafter, groups of cells were removed from the plate by chelating Ca<sup>2+</sup> with Versene, and cell number and viability were determined. Dexamethasone concentrations ranging from 10<sup>-10</sup> to 10<sup>-6</sup> M had no effect on cell number (Chart 1B) or viability (all groups were greater than 95% viable) over the entire course of the experiment. Supplementation of the growth medium with various concentrations of dexamethasone, however, results in a dose-dependent inhibition of [<sup>3</sup>H]thymidine incorporation into TCA-precipitable material (Chart 1A). The radioactivity associated with the TCA precipitates was shown to be in DNA since greater than 97% of the radioactivity could be solubilized with exhaustive DNase I digestions (data not shown). The half-maximal inhibitory concentration of dexamethasone was ~2  $\times$  10<sup>-9</sup> M, a steroid concentration which effectively saturates ~50% of the high-affinity glucocorticoid receptors found in these cells (3). Interestingly, neither the magnitude of the maximal response nor the half-maximal effective concentration of steroid changed between 1 and 4 days, suggesting that HeLa S<sub>3</sub> cells are responding rapidly to the dexamethasone supplementation.

In an attempt to determine whether the observed inhibitory action of dexamethasone on [<sup>3</sup>H]thymidine incorporation into DNA was mediated via a steroid receptor-dependent mechanism, we next examined the influence of a number of steroid hormones on HeLa S<sub>3</sub> cell growth and [<sup>3</sup>H]thymidine incorporation. As the data in Chart 2A indicate, none of the spectrum of steroids tested (cortisol, cortisone, progesterone, cortisol, estradiol) alter the growth rate of HeLa S<sub>3</sub> cells in culture under the conditions described. Of the steroids examined (Chart 2A), however, the natural glucocorticoid, cortisol, and the synthetic derivative, dexamethasone (Chart 1B), inhibit the incorporation of [<sup>3</sup>H]thymidine into DNA. The half-maximal inhibitory concentration of cortisol was ~2  $\times$  10<sup>-8</sup> M, approximately 10-fold higher than is required with dexamethasone. This value correlates well with the affinity of this natural glucocorticoid for steroid receptors and with its relative biological potencies determined in other steroid-responsive systems (7).

<sup>3</sup> The abbreviation used is: TCA, trichloroacetic acid.

Although both cortisone and progesterone, 2 known glucocorticoid antagonists, fail to elicit responses alone, both at high concentrations (10<sup>-6</sup> M) can partially block (25%) the effect of 10<sup>-8</sup> M dexamethasone on [<sup>3</sup>H]thymidine incorporation. These specificity data suggest that the action of glucocorticoids on [<sup>3</sup>H]thymidine incorporation into DNA are mediated via steroid receptor mechanisms. Furthermore, under the conditions of these experiments, we have not been able to detect any effect of dexamethasone on either [<sup>3</sup>H]leucine or [<sup>3</sup>H]uridine incorporation into protein and RNA, respectively, suggesting we are not measuring nonspecific actions of the glucocorticoids on HeLa S<sub>3</sub> (data not shown). This observation further supports the lack of glucocorticoid effect on cell multiplication observed in Charts 1 and 2.

Because maximum inhibitory effects of dexamethasone on [<sup>3</sup>H]thymidine incorporation were observed within 24 hr (Chart 1B) of exposure of cells to hormone, we sought to determine just how rapid the action of dexamethasone was on HeLa S<sub>3</sub> cells. In these experiments (Chart 3), cells were grown for various periods of time in the presence of 10<sup>-8</sup> M dexamethasone followed by a 30-min pulse of [<sup>3</sup>H]thymidine. A small but not significant dexamethasone-induced inhibition of [<sup>3</sup>H]thymidine incorporation into DNA (19%) was observed within 2.5 hr (including pulse time) of treatment with dexamethasone (paired Student's *t* test, *p* < 0.10) and a significant (*p* < 0.05) 30% inhibition of incorporation is observed by 4.5 hr of hormonal treatment. Thus, the onset of measurable effects of dexamethasone is extremely rapid in HeLa S<sub>3</sub> cells, occurring within hr of exposure of cells to steroid.

The rapid nature of the effect of dexamethasone on the inhibition of [<sup>3</sup>H]thymidine incorporation into DNA next led us to examine the dependence of this steroid hormone effect on early alterations in transcriptional and translational processes. The next experiment (Chart 4) analyzes the effects of actinomycin D, a transcriptional inhibitor, and cycloheximide, a translational inhibitor, on the action of dexamethasone in HeLa S<sub>3</sub> cells. The drugs were used at concentrations which inhibited ~90% of [<sup>3</sup>H]uridine and [<sup>3</sup>H]leucine pulse incorporation into RNA and protein, respectively. In this experiment (Chart 4), cells were exposed to steroid and/or inhibitor for a total of 6.0 hr during which time neither drug altered the viability of the cells as measured by the exclusion of trypan blue dye. In addition, cross-over experiments in which uridine incorporation was measured in the presence of cycloheximide and leucine incorporation was measured in the presence of actinomycin D, indicated only ~5% inhibitory effects for each drug under the conditions described. Both drugs themselves markedly suppressed the incorporation of [<sup>3</sup>H]thymidine in TCA-precipitable material as compared to the non-inhibitor-treated controls (Chart 4). Because of the low precursor incorporation seen in the inhibitor-treated cells, it became necessary to determine the state of thymidine pools in these cells. Table 1 shows the results of an experiment of similar design to Chart 4, which quantitates both TCA-soluble and TCA-precipitable [<sup>3</sup>H]thymidine. As in Chart 4, all cells were treated with hormone or inhibitor for only a total of 6 hr. The presence of cycloheximide (10<sup>-5</sup> M), actinomycin D (1  $\mu$ g/ml), or dexamethasone (10<sup>-6</sup> M) for this short time period reduces the intracellular acid-soluble thymidine by 34, 34, and 49%, respectively. A comparison of the effect of each inhibitor on the acid-soluble and acid-insoluble thymidine shows that while the soluble pool is

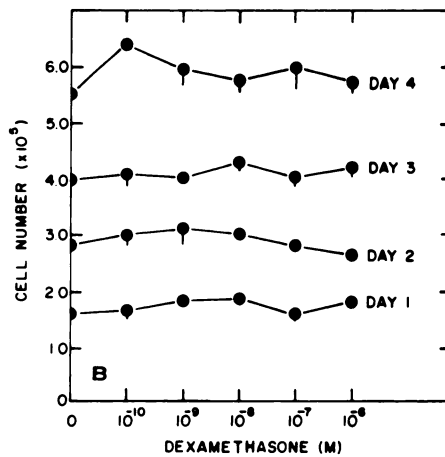
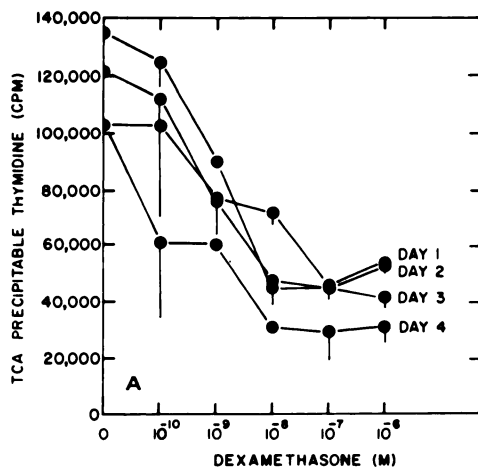


Chart 1. The influence of dexamethasone on HeLa S<sub>3</sub> cell growth and [<sup>3</sup>H]thymidine incorporation into DNA. In A, [<sup>3</sup>H]thymidine incorporation into DNA was measured after a 5-hr pulse with [<sup>3</sup>H]thymidine (2 μCi/ml) according to procedures outlined in "Materials and Methods." These measurements were made on the same cluster plates utilized for cell number and viability determinations. Points, means obtained from the cells in 2 of the wells; bars, S.D. The experimental data are representative of 3 similar experiments. In B, HeLa S<sub>3</sub> cells were plated in 24-well cluster plates at a concentration of ~1 × 10<sup>5</sup> cells/well. The cells were grown in the presence or absence of the concentrations of dexamethasone indicated. At daily intervals after plating, the cells were harvested, and viability and cell number were determined as described in "Materials and Methods." Points, mean cell numbers derived from 6 determinations in each of 2 wells; bars, S.D. The experimental data are representative of 3 such experiments performed.

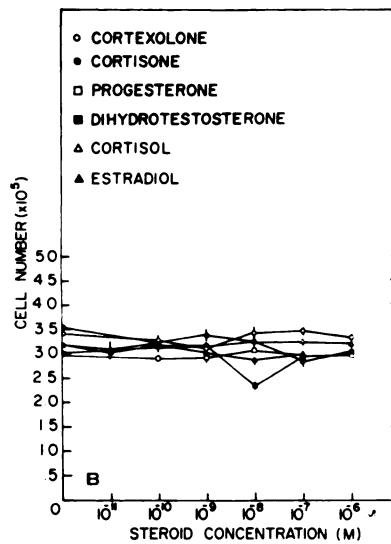
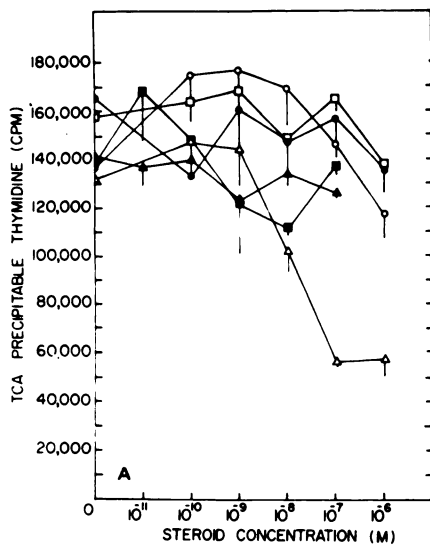


Chart 2. Steroid specificity studies on HeLa S<sub>3</sub> cell growth and [<sup>3</sup>H]thymidine incorporations into DNA. In A, [<sup>3</sup>H]thymidine incorporation was measured according to the procedures outlined in Chart 1 and "Materials and Methods." Points, means obtained from the cells in 2 wells; bars, S.D. The data was taken from a representative experiment. In B, cells were seeded as described in Chart 1 and grown in the presence of the concentrations of the steroid hormones indicated for 48 hr prior to harvesting. Cell numbers and viability were determined as in Chart 1. Points, mean cell numbers; bars, S.D.

inhibited by only ~34%, incorporation into acid-insoluble material is inhibited by greater than 90%. In cells treated with dexamethasone alone, both uptake and incorporation are inhibited by ~50%. The combined administration of glucocorticoid plus inhibitor does not lead to a further reduction in either acid-soluble or acid-insoluble material. We interpret these data to suggest that the presence of actinomycin D and cyclohexamine inhibits the accumulation of the dexamethasone-mediated process, although some caution should be taken since both drugs alter the process under investigation. More importantly, these data clearly show that dexamethasone treatment alters the acid-soluble [<sup>3</sup>H]thymidine in HeLa S<sub>3</sub>. Preliminary experiments indicate that this effect results from a combination of altered thymidine transport and increased thymidine kinase activity.

We next sought to confirm that the inhibition of [<sup>3</sup>H]thymidine incorporation into DNA in HeLa S<sub>3</sub> was a pool effect. The exogenous [<sup>3</sup>H]thymidine that we supply the HeLa S<sub>3</sub> cells would enter DNA only following transport across membranes and processing via salvage pathways (14). The study in Chart 5 examines the influence of alterations in exogenous thymidine pulse concentrations on the ability of dexamethasone to inhibit [<sup>3</sup>H]thymidine incorporation into HeLa S<sub>3</sub> DNA. The experi-

ments reported in Charts 1 to 4 all utilized ~2 × 10<sup>-8</sup> M thymidine, *i.e.*, the lowest concentration in this study. Clearly, as the thymidine concentration is increased, the magnitude of the dexamethasone effect is decreased. This observation in conjunction with our preliminary data on thymidine kinase activity and thymidine transport suggests that glucocorticoids inhibit [<sup>3</sup>H]thymidine incorporation into DNA via action on precursor pool size.

DISCUSSION

We have demonstrated that the active glucocorticoid hormones, dexamethasone and cortisol, suppress the incorporation of exogenous added [<sup>3</sup>H]thymidine into DNA in HeLa S<sub>3</sub> cells. This inhibitory effect on [<sup>3</sup>H]thymidine incorporation occurs without any alteration in the growth kinetics of HeLa S<sub>3</sub> cells. Thus, at least in HeLa S<sub>3</sub> cells, [<sup>3</sup>H]thymidine incorporation into TCA-precipitable material should not be used to index cell growth but rather as a simple monitor of cellular metabolic response. These findings represent a new effect of glucocorticoids in HeLa S<sub>3</sub> cells, one of which is probably mediated via a steroid receptor-dependent mechanism.

Our observations which demonstrate a lack of "correctness"

of [<sup>3</sup>H]thymidine incorporation into DNA are by no means new or novel. Publications suggesting a poor relationship between [<sup>3</sup>H]thymidine incorporation and cell growth have appeared in the past. Studying DNA synthesis and lymphoblast development, Youdal and MacManus (19) demonstrated that [<sup>3</sup>H]thymidine incorporation failed to accurately reflect calcium-induced DNA synthesis. Simnett and Fisher (13) reported that [<sup>3</sup>H]thymidine incorporation into DNA failed to accurately reflect the growth of lung cells in organ culture systems, when the data were compared to mitotic index determinations. In another system, the differentiating mouse mammary gland, Banerjee *et*

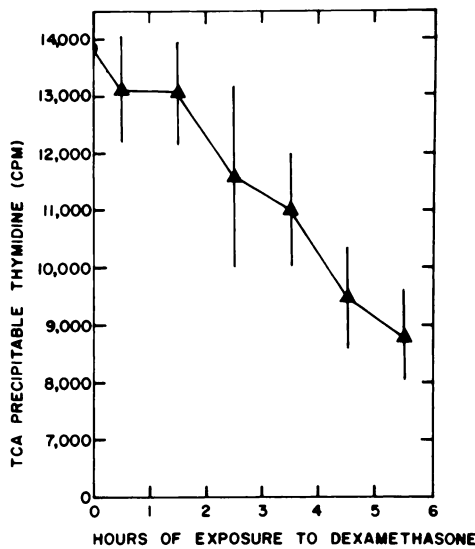


Chart 3. The rate of onset of dexamethasone effect on [<sup>3</sup>H]thymidine incorporation into DNA. HeLa S<sub>3</sub> cells were seeded in 24-well cluster plates and grown for 48 hr without dexamethasone. The medium was then supplemented with 1 × 10<sup>-8</sup> M dexamethasone by the addition of 10 μl of a stock solution of 1 × 10<sup>-6</sup> M dexamethasone prepared in Joklik's medium. Control cells received 10 μl of Joklik's medium alone. At hourly intervals thereafter, groups of wells were pulsed with [<sup>3</sup>H]thymidine (2 μCi/ml) for 30 min at 37°, and incorporation of radioactivity into TCA-precipitable material was determined as described in "Materials and Methods." Points, means derived from 4 separate experiments; bars, S.D.

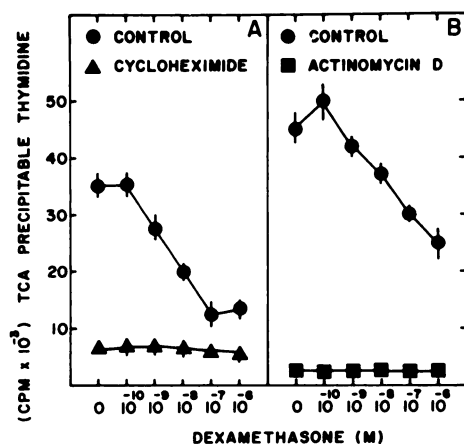


Chart 4. The effect of transcriptional and translational inhibitors or [<sup>3</sup>H]thymidine incorporation into DNA in dexamethasone-treated cells. At Time 0, cells were either exposed to various concentrations alone or in the presence of 10<sup>-5</sup> M cyclohexamide (A) or actinomycin D (1 μg/ml) (B) for a total of 5 hr. Each well was then pulsed for 1 additional hr at 37° with [<sup>3</sup>H]thymidine (2 μCi/ml), and incorporation of precursor into TCA-precipitable material was evaluated. The data plotted are the cpm of TCA-precipitable [<sup>3</sup>H]thymidine in each experimental group. Points, means obtained from 2 separate experiments; bars, range.

Table 1

The influence of dexamethasone alone and in combination with transcriptional and translational inhibitors on acid-soluble and acid-insoluble [<sup>3</sup>H]thymidine in HeLa S<sub>3</sub> cells

At Time 0, cells were exposed to 10<sup>-6</sup> M dexamethasone alone or in combination with 10<sup>-5</sup> M cyclohexamide or actinomycin D (1 μg/ml) for a total of 5 hr. Each well was then pulsed for 1 additional hr at 37° with [<sup>3</sup>H]thymidine (2 μCi/ml). Following removal of the cells from each well, groups were washed with 5.0 ml of ice-cold Joklik's medium and then precipitated with 1.0 ml of 10% TCA as described. Aliquots of the initial TCA-soluble fraction were obtained and counted, and the TCA-precipitates were washed and reprecipitated as described in "Materials and Methods." The data plotted are the cpm/2 × 10<sup>5</sup> cells accounting for dilutions in each experimental group. Values are the means ± S.E. from at least 4 separate experiments.

	Acid-soluble radioactivity	Acid-insoluble radioactivity
Control	6,892 ± 645	179,017 ± 7,729
Cyclohexamide	4,513 ± 665	13,784 ± 587
Actinomycin D	4,563 ± 746	215 ± 30
Dexamethasone	3,569 ± 701	89,880 ± 5,820
Dexamethasone, cyclohexamide	4,560 ± 225	12,792 ± 599
Dexamethasone, actinomycin D	5,457 ± 353	198 ± 4

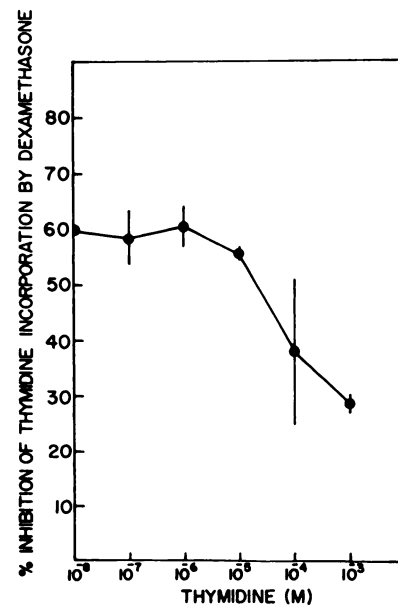


Chart 5. The influence of thymidine pulse concentration on the magnitude of the dexamethasone-induced inhibition of [<sup>3</sup>H]thymidine incorporation into DNA. HeLa S<sub>3</sub> cells were seeded in 24-well cluster plates and grown in the presence or absence of 1 × 10<sup>-8</sup> M dexamethasone for 48 hr. The cells were then pulsed for 5 hr at 37° with 2 × 10<sup>-6</sup> M [<sup>3</sup>H]thymidine (2 μCi/ml) of various specific activities, and incorporation into DNA was measured. The percentage of inhibition of incorporation produced by dexamethasone was calculated from values obtained from non-steroid-treated cells pulsed with the appropriate thymidine concentration. Points, means derived from 2 experiments; bars, S.E.

*al.* (1) demonstrated that the rate of autoradiographic [<sup>3</sup>H]thymidine incorporation did not reflect the rate of DNA synthesis. Thus, the observations made in this paper, which show that [<sup>3</sup>H]thymidine incorporation into TCA-precipitable material does not reflect cell growth, are consistent with results from several other model systems.

The data in Charts 1 to 4 strongly suggest that the action of glucocorticoids to inhibit [<sup>3</sup>H]thymidine incorporation into DNA involves the components of a steroid receptor system. The presence of specific high-affinity saturable glucocorticoid receptors in HeLa S<sub>3</sub> cells has been documented (3, 8). The HeLa S<sub>3</sub> cells that we have studied contain both cytoplasmic

and nuclear forms of glucocorticoid receptor. These cells contain ~25,000 nuclear receptors/cell and have a dissociation constant for dexamethasone of  $\sim 2 \times 10^{-9}$  M. Three additional lines of evidence suggest that the dexamethasone-induced inhibition of thymidine incorporation is a receptor-mediated process. (a) The concentration of dexamethasone necessary to achieve a 50% inhibition of [ $^3$ H]thymidine incorporation into DNA ( $1$  to  $2 \times 10^{-9}$  M) (Chart 1B) is of the same magnitude required to saturate ~50% of the nuclear glucocorticoid receptors at 37°. (b) Specificity studies (Chart 2B) show that only active glucocorticoids have the capability of inducing alterations in [ $^3$ H]thymidine incorporation. And (c) the action of dexamethasone was blocked by the transcriptional and translational inhibitors, actinomycin D and cycloheximide. Since similar observations have been made in virtually every steroid receptor system investigated (8), we are most probably studying a receptor-mediated process.

### ACKNOWLEDGMENTS

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