Regulation by Glucocorticoids of Arginase and Argininosuccinate Synthetase in Cultured Rat Hepatoma Cells*

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We have examined and characterized the regulation by glucocorticoids of the levels of arginase and argininosuccinate synthetase in two rat hepatoma cell lines (H4-II-E-C3) and MH1C1. Hydrocortisone elevates the activity of both enzymes in a time- and dose-dependent fashion. This effect was blunted markedly by small amounts of ethanol (0.1 to 0.9% [v/v]) and blocked substantially by a high molar excess of the "anti-inducer" steroid fluoxymesterone. The other "optimal" inducers dexamethasone and corticosterone were as effective as hydrocortisone in elevating the levels of these enzymes at saturating concentrations. Inhibition of these stimulations by cycloheximide indicated that ongoing cellular protein synthesis was required for both effects, and the admixture of extracts from fully stimulated and basal cells gave no evidence for the existence of direct inhibitors or activators of either enzyme. The results corroborate findings from earlier whole-animal studies and provide evidence for the following conclusions. (i) This stimulation by hydrocortisone of urea-cycle enzymes in the cultured hepatoma cells is mediated by a classical glucocorticoid mechanism involving initial binding to specific cytoplasmic steroid receptors and the eventual accumulation of new enzyme molecules. (ii) These cell lines thus constitute valid experimental models for use in further detailed studies on the molecular mechanism(s) through which glucocorticoids and intermediary metabolites effect a selective modulation of arginase and argininosuccinate synthetase gene expression in the differentiated mammalian liver.

The urea cycle is the main biochemical pathway for the detoxification of ammonia in mammals, being found in its entirety only in liver (1). In this 5-step process, 1 molecule of urea is ultimately generated from 2 of ammonia and 1 of carbon dioxide. After the initial synthesis of carbamyl phosphate and condensation of the latter with ornithine to form urea and regenerating ornithine for the next round of reactions, the mitochondrial enzymes are confined to liver, whereas the cytoplasmic ones are found in low amounts in a variety of other tissues (1).

To date, inheritable abnormalities in all 5 of these enzymes have been described in man, and in almost all instances such metabolic disorders have been accompanied by neurologic impairment and mental retardation (2). Since among these cases, 10 individuals with arginase (2-6) and 14 with argininosuccinate synthetase (2) deficiencies have been reported, an elucidation of the mechanisms regulating the levels of these two enzymes in the normal liver would seem of primary interest.

In 1963 Schimke (7) reported that the administration of pharmacologic doses (25 mg/kg) of cortisone to intact or adrenalectomized rats caused elevations in the activity of all 5 enzymes of the urea cycle and a concomitant increase in urea production, thus providing preliminary evidence that glucocorticoids regulate the steady state levels of these gene products in vivo. In an effort to investigate further this possibility, we wished to use mammalian cell lines as a simplified means of elucidating the molecular bases for the regulation of the urea cycle by hormones and other factors. In this regard, Shimanke also discovered that arginase and argininosuccinate synthetase and lyase were present in the human cell line HeLa (8). Since, however, HeLa was originally derived from a carcinoma of the uterine cervix (9), we questioned whether or not the modulation of gene expression in those cells would reflect the situation in liver. Moreover, with another mammalian line that did in fact originate from rat liver and contained physiologically significant levels of all 5 urea-cycle enzymes (the 7800C hepatoma cells), the addition of glucocorticoids to the culture medium had no effect on the activity of any one of these gene products (10). We found, in contrast, that two rat hepatoma lines (H4-II-E-C3 and MH1C1), studied for a number of years in our laboratory, contained arginase and argininosuccinate synthetase at low constitutive levels and that these activities were markedly elevated after exposure of the cells to glucocorticoid hormones. We report here the detailed characterization of this stimulation by the "optimal-inducer" steroid, hydrocortisone, and demonstrate that the hormone acts in the H4 cells through a classical glucocorticoid mechanism.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—Stock surface cultures of the continuous rat hepatoma lines H4-II-E-C3 (11), MH1C1 (12), and HTC (13) were plated and grown in modified Swim's Medium S-77 supplemented with 5% fetal bovine serum. They were passaged at a ratio of 1:3 every 3 to 4 days, and subcultures were grown in T-25 flasks. The details of all reagents and protocols used have been described previously (14-18). Additions of glucocorticoids were by a concentration of 10 μg/ml in 0.9% [v/v] ethanol. The abbreviations and trivial names used are: H4, H-4-II-E-C3; corticosterone, preg-4-enediol-3,20-dione; hydrocortisone, sodium 17α-hydroxycorticosterone 21-succinate; dexamethasone, sodium 9a-fluoro-11β,17α,21-trihydroxy-16α-methylpregn-1,4-diene-3,20-dione; fluoxymesterone, 9α-fluoro-11β-hydroxy-17α-methylandrost-4-ene-17α-ol-3-one; cycloheximide, 3-[2-(3,5-di methyl-2-oxocyclobexyl)-2-hydroxyethyl]glutarimide.

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(v/v) fetal or neonatal bovine serum plus 10% adult equine serum (standard medium), as described previously (14, 15). Along with other liver-associated gene products (11, 12), the first two lines contain the hepatic enzyme phenylalanine hydroxylase at constitutive levels that are markedly elevated by exposure of the intact cells to glucocorticoids (14-17) and/or charcoal-extracted (i.e. glucocorticoid-free) serum (16). Phenylalanine hydroxylase, however, has never been detected in HTC cells (14, 17).

**Experimental Incubations**—Replicate cultures for enzyme assay were initiated in 75-cm² (Lux) flasks and grown in standard medium for 3 or more days to a confluent population density. Cells were washed twice with serum-free (basal) 5-77 medium before exposure to experimental media. Horseradish peroxidase was extracted thrice with charcoal at 45 °C to remove all endogenous glucocorticoids (16), filter sterilized, and stored at -20 °C until used. Experimental media were changed every 2 to 3 days and always on the day before harvesting.

When parallel 25-cm² cultures were pulse-labeled with [³⁵S]methionine (specific radioactivity, 335 Ci/mol; New England Nuclear) for measurement of their relative rates of general protein synthesis, portions of the hydrocortisone- and cycloheximide-containing media were also prepared in bulk at the start of the incubation period and were kept overnight at 37 °C along with the cultures. A nutrient renewal with these prewarmed experimental media was then given to all cultures at the time of isotope addition in order to avoid indeterminate changes in the final specific radioactivity of the precursor as a result of metabolism by the cells of the [³⁵S]methionine in the culture medium (16) during the initial overnight incubation. Precipitation of cellular proteins in situ with trichloroacetic acid and measurement of the incorporation of radioactivity into acid-soluble and-insoluble material were performed as reported previously (Method B, Ref. 16). Preparative Cell Extracts and Assays for Arginase and Argininosuccinate Synthetase—Cells were detached by trypsin and extracts prepared by detergent treatment essentially as described previously for measurement of their phenylalanine-hydroxylase levels (14, 15). However, for assay of arginase and the synthetase, the procedure was scaled down and extracts were diluted in the following manner. The cells suspended from a single flask were washed once with Buffer A (0.15 M KC1, 10 mM Tris-HCl, pH 7.5) by centrifuging and then transferred to a tared plastic 1.5-ml Eppendorf Microfuge tube in about 1 ml of that same buffer. After removal of a drop of this suspension for verification of plasma-membrane integrity by trypan blue dye exclusion, the cells were sedimented at 500 × g and room temperature for 10 min. The resulting pellet was weighed, and the cells were disrupted by a 15-min exposure to Triton X-100 (0.4% (v/v) in Buffer A) at 0 °C in a weight to volume ratio of 250 mg of packed cells/ml (15). Enzymic activities were determined in triplicate in a crude supernatant fraction (12,800 × g, 15 min or longer) of the cell lysate after dilution with Buffer B (1.0 mM Tris-HCl, pH 7.8), usually 1/25 for arginase and 1/250 for the synthetase. Whereas arginase was assayed on the day of harvest, the synthetase was normally measured after storage at -70 °C from for a few days to several weeks. After either interval at that temperature, activities were stable, although somewhat reduced, upon a single freezing and thawing. Proteins, however, tended to aggregate after prolonged freezing to an extent prohibitive for their determination by our standard method of dye binding (18, 19). Thus, except in the earliest experiments, aliquots of extracts to be assayed for protein were kept at 4 °C and processed within a few days.

Arginase was measured by the method of Spector et al. (20), a two-step procedure involving the initial conversion of [guanido-¹⁴C]arginine to [¹⁴C]urea by the proband samples (15 to 30 pg of protein) and the subsequent liberation of ¹⁴CO₂ from the reaction product by the proband samples (15 to 30 pg of protein) and then transferred to a tared plastic 1.5-ml Eppendorf Microfuge tube in about 1 ml of that same buffer. After removal of a drop of this suspension for verification of plasma-membrane integrity by trypan blue dye exclusion, the cells were sedimented at 500 × g and room temperature for 10 min. The resulting pellet was weighed, and the cells were disrupted by a 15-min exposure to Triton X-100 (0.4% (v/v) in Buffer A) at 0 °C in a weight to volume ratio of 250 mg of packed cells/ml (15). Enzymic activities were determined in triplicate in a crude supernatant fraction (12,800 × g, 15 min or longer) of the cell lysate after dilution with Buffer B (1.0 mM Tris-HCl, pH 7.8), usually 1/25 for arginase and 1/250 for the synthetase. Whereas arginase was assayed on the day of harvest, the synthetase was normally measured after storage at -70 °C from for a few days to several weeks. After either interval at that temperature, activities were stable, although somewhat reduced, upon a single freezing and thawing. Proteins, however, tended to aggregate after prolonged freezing to an extent prohibitive for their determination by our standard method of dye binding (18, 19). Thus, except in the earliest experiments, aliquots of extracts to be assayed for protein were kept at 4 °C and processed within a few days.

When it was desirable to represent in a single figure the data from separate experiments in which the basal and optimally induced cultures exhibited enzyme levels that were not respectively identical, all values were normalized in the following manner. The increase in enzymatic activity above basal levels was first determined by subtracting the value for the fully stimulated cultures, arbitrarily chosen to be those exposed to 1.0 μM hydrocortisone for 3 days or longer. The corresponding figures for all other groups within the same experiment were then expressed as a per cent of this maximum and were finally plotted on the ordinate (cf. Figs. 3-6).

Proteins were estimated by a method of dye binding, either as described by BioRad or as modified from the original publication (18) in a later report (19). Radioactivity was measured by liquid scintillation spectrometry with 10 ml of a 3a70B liquid fluor (Research Products International, Mt. Prospect, IL) in a Beckman Model LS320 counter, for enzyme assays, and with 10 ml of either Scintiverse (Fisher) or Hydrofluor (National Diagnostics, Somerville, NJ) mixture as described earlier (14, 16), for [¹⁴C]leucine incorporation into proteins and acid-soluble products.

**RESULTS**

Effect of Hydrocortisone, Serum, and Culture Density on Arginase Gene Expression in Cultured Rat Hepatoma Lines—Treatment of H4 cells with 1.0 μM hydrocortisone for 3 days in the presence or absence of serum caused a marked increase (about 10-fold) in arginase activity (Fig. 1). The enzyme activity was elevated regardless of whether the data were chosen to reflect the total arginase content per culture (i.e. the activity per assay tube) or were normalized to specific enzyme levels per mg of cellular protein. Although actual
of arginase that were elevated 2- to 3-fold after exposure to 1.0 μM hydrocortisone (Experiments A and C, Table I). Moreover, with these cells, as with the H4 cells, arginase gene expression was not influenced by the extent of cell crowdedness: in Experiments B and C of Table I, arginase specific activities in either the basal or the induced cultures did not vary significantly when the final population densities differed by as much as 4- to 5-fold. By contrast, the HTC line was devoid of detectable arginase activity under all conditions tested (Experiment A, Table I). The H4 cells were thus chosen over the MH,C line cells for further characterization of the effect of glucocorticoids on arginase.

Examination of two other permanent rat-hepatoma lines (MH,C, and HTC) for arginase activity

Experiment A: Replicate cultures of each cell type were washed free of serum and then exposed to S-77 medium either with or without 1.0 μM hydrocortisone. A medium renewal was provided two days later, and the cultures were harvested for arginase assay after a 48-h exposure to the experimental media. At this time, the H4 cells were confluent; the HTC cells were postconfluent; and the MH,C cells, which never attain complete confluency, covered about two-thirds of the substratum. Experiment B: Two sets of replicate cultures were initiated in parallel from H4-11-E-C3 stock cultures at an 8-fold difference in cell inoculum, with 8 flasks at the lower density and 4 at the higher. Three days later, after being washed free of serum, one-half of each set received basal medium alone, while the other half was given medium plus 1.0 μM hydrocortisone. A medium renewal was provided on the fifth day, and the cultures were harvested for arginase assay after a 65-h exposure to the experimental media. At that time, the denser cultures were just subconfluent; while the sparser cultures were at 18% (stimulated) to 35% (basal) of that density, as judged by the total wet weight of cell material harvested per flask. Experiment C: Two sets of 4 replicate cultures were initiated in parallel from MH,C stock cultures at a 6-fold difference in cell inoculum. On the following day, after being washed free of serum, one-half of each set received S-77 medium alone, while the other half was given medium with 1.0 μM hydrocortisone. The cultures were harvested for arginase assay 96.5 h later with no intervening medium renewal. At that time, the high density cultures were about two-thirds confluent; while the low density cultures were at 27% (stimulated) to 35% (basal) of that density, as judged by the total wet weight of cell material harvested per flask.

**Table 1**

**Examination of two other permanent rat-hepatoma lines (MH,C, and HTC) for arginase activity**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Growth status</th>
<th>Hydrocortisone</th>
<th>Arginase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H4-11-E-C3</td>
<td>H</td>
<td>−</td>
<td>23.4, 22.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>122, 114</td>
</tr>
<tr>
<td>B</td>
<td>MH,C</td>
<td>H</td>
<td>−</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>20.7</td>
</tr>
<tr>
<td>C</td>
<td>HTC</td>
<td>H</td>
<td>−</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>B</td>
<td>H4-11-E-C3</td>
<td>L</td>
<td>−</td>
<td>20.0, 23.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>138, 156</td>
</tr>
<tr>
<td>C</td>
<td>MH,C</td>
<td>L</td>
<td>−</td>
<td>22.8, 24.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>125, 141</td>
</tr>
<tr>
<td>B</td>
<td>H4-11-E-C3</td>
<td>L</td>
<td>−</td>
<td>27.8, 31.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>67.3, 82.0</td>
</tr>
<tr>
<td>C</td>
<td>MH,C</td>
<td>H</td>
<td>−</td>
<td>35.2, 36.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>70.7</td>
</tr>
</tbody>
</table>

*H, high density; L, low density.

Each determination is for an individual culture.

*Not detectable.

Examination of two other permanent rat-hepatoma lines (MH,C, and HTC) for arginase activity

Glucocorticoids Control Urea Cycle Enzymes in Hepatoma Cells

FIG. 2. Kinetics of stimulation of arginase in H4 cultures exposed to hydrocortisone. Replicate postconfluent cultures of H4 cells were washed free of serum and were then given S-77 medium with or without 1.0 μM hydrocortisone, either directly (Experiment 2) or after a 2-day preincubation in the basal medium alone (Experiment 1). In all three experiments, hydrocortisone was added to duplicate cultures at the interval before harvesting indicated on the abscissa so that all cultures could be processed on the same day. Thus, in Experiment 1, 2, and 3 all cultures were assayed 5, 3, and 6 days after serum withdrawal, with the basal cultures having arginase activities (mean ± one-half range or S.D. (number of cultures)) of 146 ± 49 (3) munits/tube, 12.0 ± 1.9 (2) units/mg, and 17.0 ± 2.0 (4) units/mg, respectively. On the ordinate, all values within each experiment are plotted as fold increase above the mean basal activity (set at 1.0), all data being treated as in Fig. 1. In Experiment 3, 20% (v/v) charcoal-extracted horse serum was added to some pairs of cultures, either alone (C) or with 1.0 μM hydrocorti- sone (M) 2 days before harvesting. The undiluted crude extracts from these cultures and from the basal and 2-day hydrocortisone-stimu- lated cultures of this experiment were used for the measurement of phenylalanine-hydroxylase content as cited under "Experimental Procedures." In contrast to its inability to stimulate arginase activity in either the absence or the presence of hydrocortisone, this same preparation of glucocorticoid-free serum retained its capacity to ele-
of hydrocortisone on arginase activity because of their faster growth rate and higher enzyme content.

**Dynamics of Stimulation by Hydrocortisone of Arginase Activity in H4 Cells**—Arginase levels began to increase after H4 cells had been treated with hydrocortisone (1.0 μM) for 8 to 10 h, while the maximal elevation of enzyme content occurred at least 3 days (Fig. 2). A similarly long time course had been observed for the restoration of normal hepatic arginase levels in vivo in hormone-replacement studies with adrenalectomized animals (7). Although a lag period of greater than 1 h is typical of glucocorticoid-modulated processes (13, 14, 23, 24), the rise in arginase activity in the H4 cells caused by hydrocortisone was slower than the increase in their tyrosine aminotransferase content reported previously (24). The kinetics were more akin to those seen with the stimulation of the phenylalanine-hydroxylase levels in cells under comparable conditions (14). The decay of arginase activity after withdrawal of hydrocortisone from fully stimulated cultures was also relatively slow (Fig. 3); the half-life for this process being about 3 days. We have as yet no explanation for the fact that the kinetics of this decrease appear to be linear rather than exponential, in contrast to the dynamics of the declines in tyrosine aminotransferase (24, 25) or phenylalanine hydroxylase (16) in these same cells after removal of glucocorticoid or native serum, respectively.

Over a 3-day induction period, the concentration of hydrocortisone needed to elicit a detectable increase in arginase activity was between 10 and 25 nM (Fig. 4); half-maximal stimulation was seen at about 50 nM; and maximal values were attained at between 100 and 250 nM. This dose dependency was nearly identical with those observed earlier for the stimulatory effects of hydrocortisone on tyrosine-aminotransferase activity in HTC cells (26) and on phenylalanine-hydroxylase levels in the H4 cells (14). In all three of these examples, a maximal hormonal effect was achieved at glucocorticoid concentrations within the physiologic range (circulating levels in man, 30 to 700 nM (27); measured concentration...
effective concentration (1.0 μM), elevated cellular arginase levels with a potency equal to that of hydrocortisone, provided that the same amounts of ethanol were present in the cultures being compared (Fig. 5). Moreover, fluoxymesterone, at a high concentration (10 μM), showed no stimulation and completely blocked the action of a near-saturating concentration (100 nM) of hydrocortisone (Fig. 5A). The results from a further experiment demonstrated that this antagonism by fluoxymesterone occurred in a competitive fashion (Fig. 6A). A replotted

![Graph](image_url)

**Fig. 5.** Steroid specificity in the modulation of arginase levels in H4 cells. Replicate confluent cultures of H4 cells were washed free of serum, were transferred directly to S-77 medium containing the additions indicated in the panel keys, and were harvested for concentration of arginase activity. The concentration of hydrocortisone (Fig. 5A) and fluoxymesterone (Fig. 5B) expressed as a per cent of the value obtained upon stimulation with 1.0 μM hydrocortisone alone (24 ± 8 (A) and 19 ± 2 (B) units/mg), is plotted (ordinate) versus molar concentration of the optimal inducer examined (abscissa; note discontinuities in scale), either with or without 10 μM fluoxymesterone.

![Graph](image_url)

**Fig. 6.** Competitive antagonism by fluoxymesterone of the stimulation by hydrocortisone of H4-cell arginase activity. Replicate confluent cultures were exposed to experimental media of composition indicated in the figure key as described in the legend to Fig. 5. Ethanol was present at a concentration of 0.1% (half-open symbols) or 0.9% (open symbols) of the stimulated levels of this enzyme. The time (Fig. 7A) and dose (Fig. 7B) dependencies of the hormonal effect were comparable to those seen with arginase (cf. Figs. 2 and 4). Moreover, as had also been found with arginase, the results

![Graph](image_url)

**Fig. 7.** Dynamics of stimulation of argininosuccinate synthetase in H4 cells exposed to hydrocortisone. A, kinetics: the frozen extracts from Experiment 3 of Fig. 2 were thawed and assayed for the synthetase. Enzymatic activity (units/mg), is plotted (ordinate) as a function of the time (abscissa) either with or without ethanol: the frozen extracts from Experiment 2 of Fig. 4 were thawed and assayed for the synthetase. Enzymatic activity (units/mg), is plotted (ordinate) as a function of duration of exposure to hydrocortisone and/or charcoal-extracted serum (abscissa), the symbols being the same as in Fig. 2. B, dose-response in the absence or presence of ethanol: the frozen extracts from Experiment 2 of Fig. 4 were thawed and assayed for the synthetase. Enzymatic activity (units/mg), is plotted (ordinate) as a function of hydrocortisone concentration (abscissa), the symbols being the same as in Fig. 4. The X represents the mean ordinate value, and each adjoining bracket the S.D., for the combined data from the cultures receiving between 75 nM to 1.0 μM hydrocortisone without ethanol.

This reversible agonism/antagonism presumably results from competition between the two molecules for binding to specific cytosolic glucocorticoid receptors, as has been well established in other systems (29): since receptor occupancy by fluoxymesterone fails to form the activated receptor-ligand complex normally generated by the binding of an optimal inducer and required for stimulation to occur, the anti-inducer not only exhibits no positive action of its own but also prevents the formation of fruitful complexes between the receptors and hydrocortisone.

**Effect of Glucocorticoids on Argininosuccinate Synthetase Levels in H4 Cells—** Hydrocortisone also increased markedly the activity of argininosuccinate synthetase in the H4 cells, while charcoal-extracted serum affected neither the basal nor the stimulated levels of this enzyme. The time (Fig. 7A) and dose (Fig. 7B) dependencies of the hormonal effect were comparable to those seen with arginase (cf. Figs. 2 and 4). Moreover, as had also been found with arginase, the results

*With the kind assistance of Dr. James F. McGinnis, we have measured glucocorticoid binding by the H4 cells after growth to confluency in the standard medium and have found their cytoplasmic receptor content to be about 45,000/ng of total cell protein or 8000/cell.
Hydrocortisone 1.0
Corticosterone 1.0
exposed to the indicated agents for 63.5 h before harvesting for
Hydrocortisone 1.0
Dexamethasone 1.0
synthetase assay.

or left unmixed, were kept for 60 min before assay at the temperature
stimulatory or inhibitory interaction had taken place during the
either individually or after admixture in a 1:1 volume ratio. At this
assayed for phenylalanine hydroxylase: the values obtained with the
an aliquot from each of the unmixed, unincubated extracts was also

Steroid specificity in the modulation
indicated in the second column. As a further corroboration that the
fully induced and basal cells were 720 and
replicate postconfluent cultures of H4 cells were washed free of
arginase  and

\*Admixture of equal amounts of both extracts in vitro.

TABLE II
Steroid specificity in the modulation of H4-cell argininosuccinate-synthetase levels: effects of alternative optimal inducers and an anti-inducer

<table>
<thead>
<tr>
<th>Nature</th>
<th>Concentration</th>
<th>Hydrocortisone</th>
<th>Ethanol</th>
<th>Argininosuccinate Synthetase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>10 µM</td>
<td>0.1% (v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;c</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1140 ± 61</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
<td>3880 ± 25</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>1480 ± 1</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.0</td>
<td>-</td>
<td>+</td>
<td>1890 ± 176</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>900 ± 565</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>8580 ± 806</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>9040 ± 839</td>
</tr>
</tbody>
</table>

In a single experiment, replicate postconfluent cultures were exposed to the indicated agents for 63.3 h before harvesting for synthetase assay.

Mean value from duplicate cultures ± S.D.

Not added.

TABLE III
Effect of admixture of extracts from fully induced and basal H4 cells on their arginase and argininosuccinate-synthetase activities

Replicate postconfluent cultures of H4 cells were washed free of serum and transferred directly to basal medium either with or without 1.0 µM hydrocortisone. After a 64-h exposure to these media, the cultures were harvested for enzymatic assay and the 12,800 x g supernatant fractions handled as follows. Aliquots of the extracts from the fully induced and basal cultures were assayed immediately, either individually or after admixture in a 1:1 volume ratio. At this time, some of the basal extract was assayed in the presence of added hydrocortisone at the final concentration indicated in the third column. Other portions of both extracts, either mixed in the same fashion or left unmixed, were kept for 60 min before assay at the temperature indicated in the second column. As a further corroborating that the cells retained their sensitivity to hydrocortisone in this experiment, an aliquot of each of the unmixed, unincubated extracts was also assayed for phenylalanine hydroxylase; the values obtained with the fully induced and basal cells were 720 and 66 units/g wet weight, respectively. The numbers within parentheses are the enzyme activities that were expected to be found in the mixed samples if no stimulatory or inhibitory interaction had taken place during the interval between admixture and assay.

<table>
<thead>
<tr>
<th>Cellular status</th>
<th>Treatment in vitro</th>
<th>Catalytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation temperature</td>
<td>Arginase</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>units/mg of protein</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>4.7</td>
</tr>
<tr>
<td>B</td>
<td>1.0</td>
<td>4.7</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>4.5</td>
</tr>
<tr>
<td>I</td>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>I + B'</td>
<td>27.7 (22.6)</td>
<td>610</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>5.3</td>
</tr>
<tr>
<td>I</td>
<td>0.5</td>
<td>49.5</td>
</tr>
<tr>
<td>I + B</td>
<td>35.2 (27.4)</td>
<td>570</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>5.5</td>
</tr>
<tr>
<td>I</td>
<td>0.5</td>
<td>38.3</td>
</tr>
<tr>
<td>I + B</td>
<td>23.4 (21.9)</td>
<td>380</td>
</tr>
</tbody>
</table>

I = fully induced; B = basal.
Mean ± S.D.
Not incubated; assayed directly.
Admixture of equal amounts of both extracts in vitro.
Value predicted on the basis of simple numerical activity.
from a single additional experiment indicated that either dexamethasone or corticosterone was as potent as hydrocortisone as an alternative inducer of the synthetase and that the anti-inducer flutamide could effectively block the stimulatory action of the latter steroid (Table II).

**INITIAL INVESTIGATIONS INTO THE MECHANISM OF ACTION OF HYCROCORITISONE**—A mixing experiment was performed in order to examine the possibility that hydrocortisone might stimulate cellular arginase activity by activating the enzyme, either directly or indirectly (Table III). The admixture of extracts from fully stimulated and basal cultures yielded specific activities for arginase and for the synthetase that were within the range expected on the basis of simple numerical additivity, even after being kept at 0 or 37 °C for 60 min. We thus gained no evidence for the presence of a soluble inhibitor of these enzymes in basal cells or for the existence of an enzymatic activator in stimulated cells. Moreover, hydrocortisone (1.0 to 100 µM), when included in the assay in vitro, failed to increase the low activity of either enzyme in extracts from the basal cultures. Finally, the induction of arginase (Fig. 8A) or the synthetase (Fig. 8B) by hydrocortisone required the continuation of general protein synthesis during the incubation period. This observation is consistent with, though does not prove, the notion that the hormone acts by increasing the number of enzyme molecules rather than by some form of direct enzymic activation.

**DISCUSSION**
To date, studies on the regulation of arginase activity in cell-culture systems have, with few exceptions (8), emphasized aspects of the cell and molecular biology of this enzyme other
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than its role in the control of urea production (30–32). Through the use of permanent cell lines lacking the complete urea cycle and other liver-associated functions, these investigations, however, did reveal that cellular arginase activity was stimulated by increased concentrations of arginine (the enzyme's substrate) and depressed by elevated levels of proline (a product or ornithine metabolism outside the urea cycle) in the culture medium (31, 32).

We have recently reported the discovery of an immunologically distinct form of arginase in the kidney of a patient deficient in liver-arginase activity (33). The likelihood of the existence of a second arginase genetic locus in man, suggested by this finding, thus raises important questions regarding the evolution and function of the two enzymic forms and underscores the importance of the issue of organotypic specificity in the choice of a model system for studying the relationship of arginase to the urea cycle. Because the objective of the work reported here has been to characterize a liver-derived cell-culture system for this specific purpose, we are encouraged to have found that two permanent rat hepatoma lines, already at our disposal, contain arginase and argininosuccinate synthetase as constitutive enzymes whose activities are elevated to physiologic levels upon exposure of the cells to glucocorticoid hormones. Since earlier steroid-replacement experiments with adrenalectomized rats had suggested the possibility that this hormonal control was also occurring in vivo, the correlation of those observations with the results with the cultured cells not only provides further support for the conclusions drawn from the whole-animal studies but also reassures us that the hepatoma cells continue to "breed true" with respect to the phenotype of the hepatic cells from which they were originally derived. That this phenotypic fidelity could not be assumed to obtain for every liver-derived permanent cell line was indicated by the absence of sensitivity to glucocorticoids in hepatoma lines of rat (7800C1, Ref. 10) and human (HEPG2 (34)) origin with respect to the expression of either of the enzymes. Few of these two enzymic activities: in each of these examples the enzyme contents were found to be constitutively high. Conversely, in 2-day-old primary cultures of diploid rat hepatocytes, dexamethasone exerted only a so-called "permissive" effect on the stimulatory action of glucagon with respect to all 5 urea-cycle enzymes: the glucocorticoid caused no significant elevation in the level of any one of these if present alone but caused 1.5- to 2.5-fold increases in their activities by 24 h when added in combination with glucagon, the latter exhibiting only a modest stimulation on its own under these conditions. Longer periods of exposure to the agents were not examined; and it is noteworthy that these effects were not observed with conventional monolayer cultures of the hepatocytes, but were only obtained under conditions of steady state "perfusion," in which fresh experimental medium was continuously added to the incubation vessel in an open circulation system (35). From these results, it would thus appear that the H4 cells are more sensitive to glucocorticoid than are their clonal derivatives and the rate of albumin synthesis in the MI,H4 cells (12) are also under control by glucocorticoids. Indeed, this class of steroid hormone evokes a plethora of responses in the adult mammalian liver, including the induction of a gaggle of cytoplasmic enzymes (42, 43) and even a modest increase in the rate of macromolecular synthesis (42, 44). The elucidation of differences, if any, among the mechanisms of these hormonal actions at the biochemical-genetic level must, however, await the detailed characterization of the molecular biology involved in each individual situation. In this regard, we are at present conducting parallel studies on the molecular genetics of the induction by hydrocortisone of the two urea-cycle enzymes and of phenylalanine hydroxylase in the H4 cells. Although the results reported here have indicated that the steroid specificities and biochemical dynamics of the hormone responses on the part of these three gene products were similar, it has yet to be determined whether or not the elevations in the levels of arginase and the synthetase under these conditions, like the stimulation of cellular phenylalanine-hydroxylase content, are mediated by commensurate increases in the rate of enzyme synthesis (45) and in the level of specific messenger-RNA translational activity associated with polysomes (46).

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Glucocorticoids Control Urea Cycle Enzymes in Hepatoma Cells


Regulation of glucocorticoids of arginase and argininosuccinate synthetase in cultured rat hepatoma cells.

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