Phenylalanine Hydroxylase in Cultured Hepatocytes

I. HORMONAL CONTROL OF ENZYME LEVELS*

(Received for publication, July 10, 1972)

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SUMMARY

The presence of phenylalanine hydroxylase as a constitutive enzyme in two clonal cell lines (H4-II-E-C3 and MH1C1) derived from rat hepatomas and cultured in vitro is described; eight other clonal cell strains originating from rat or rabbit liver did not contain detectable amounts of this enzyme. The levels of phenylalanine hydroxylase in the two hepatoma cell lines were stimulated by hydrocortisone, corticosterone, dexamethasone or N6-O'′-dibutyryl-3′,5′-cyclic adenosine monophosphate. The stimulation of the levels of the enzyme by hydrocortisone in the H4-II-E-C3 line was shown to depend on de novo protein synthesis as it was inhibited by cycloheximide. It is postulated that the hepatoma cell lines are probably good models for studying the factors regulating the expression of the phenylalanine hydroxylase gene.

The genetic and epigenetic factors governing the regulation of phenylalanine hydroxylase activity in the normal differentiated mammalian hepatocyte have not yet been defined; nor have the molecular mechanisms responsible for the apparent absence of this enzyme in the liver of "classical" phenylketonuric phenotype been determined. This is the first report on the presence of phenylalanine hydroxylase in cultured cells originating from rat hepatomas and cultured in vitro.
All cells were grown in surface culture in Falcon T-30 or T-250 flasks, the medium being changed thrice weekly. Cultures were maintained at 37° in a 93% air and 7% CO₂ atmosphere in a Napco model 392 incubator (Scientific Products, 17111 Red Hill Ave., Santa Ana, Calif. 92705). Samples of all cell lines were frozen and stored in a liquid-nitrogen freezer in their respective growth media supplemented with 10% (v/v) dimethyl-sulfoxide.

Subcultivations were carried out as follows. After two washings with citrate-saline solution followed by a single rapid washing with the appropriate trypsin solution, the cells of all cell lines, except H4, were detached from the growth surface of the flasks by a 5-min incubation at 37° with a 0.1% (w/v) solution of trypsin in citrate-saline solution. The cell suspension was then layered on top of 10 ml of growth medium and the cells, sedimented by centrifuging through the medium, were dispersed in a convenient volume of fresh medium and inoculated into new culture vessels. The H4 cells were treated with a 0.025% trypsin solution for 5 min at 37°.

All cell lines were tested, periodically, for bacterial or fungal contamination by inoculation of spent culture media into thiglycylate broth. In addition, the HTCC, RLC, H4, and MHCl cells were assayed for the presence of mycoplasma with NABI (North American Biologicals, Inc., 13233 S. Normandie Ave., Cardena, Calif. 90249), or GIBCO (Grand Island Biological Co., 2323 Fifth St., Berkeley, Calif. 94710) mycoplasma broth and agar plates. All tests for microorganisms were negative.7 In order to reduce the likelihood of inadvertent contamination with mycoplasma through normal handling procedures, stock cultures of HTC, RLC, H4, and MHCl cells were routinely maintained and passaged continuously in T-30 flasks by disposable plastic syringes without pipetting by mouth.

Unless otherwise indicated, H4 cells were plated and cultivated for experiments as follows. Confluent populations of cells, grown in T-250 flasks, were subcultured for the experiments at a 1:10 or 1:15 dilution and seeded into fresh flasks each containing 20 ml of growth medium; a fresh change of medium was given before or after the receipt of the prototype cultures in our laboratory. The viral infection is not related to the presence or absence of phenylalanine hydroxylase activity within 2 to 3 hours of this step; their protein content was measured by the method of Lowry et al. (9) with bovine serum albumin as a standard. In order to gain evidence that the recoveries of phenylalanine hydroxylase enzyme by these procedures were complete, in several experiments treatment with trypsin (0.025% (w/v), 37°, 5 min) and Triton X-100 (0.1% (v/v), 4°, 15 min) were chosen as alternatives to mechanical harvesting and cell disruption by ultrasound. Since these methods resulted in no significant difference in the enzyme yield, we concluded that the mechanical harvesting and ultrasonic disruption of cells gave a satisfactory estimate of total cellular phenylalanine hydroxylase content; all data reported were obtained on preparations made by the mechanical processes.

In all experiments in which the phenylalanine hydroxylase levels of cultured cells were compared with those of extracts of adult rat liver, the animals were killed by cervical dislocation followed by decapitation; 1.0 g of liver was immediately excised.

Preparation of Extracts of H4-T1-E-C3 Cells and Rat Liver for Phenylalanine Hydroxylase Assay—Each experimental group consisted of 10 T-250 flasks of cells grown to confluency. The experimental media were removed by aspiration, and the cell sheets in each flask were washed twice with 10 ml of citrate-saline solution. Three milliliters of cold Ca++- and Mg++-free phosphate-buffered saline (NaCl, KCl, NaHPO₄, KH₂PO₄; 8.0, 0.2, 2.17, and 0.2 g per liter) were then added to each flask and the cells were detached mechanically by passing a rotating 2-cm long cylindrical Teflon-coated magnetic stirring bar back and forth over the vessel growth surface. The suspended cells from each experimental group were combined and transferred to a plastic conical centrifuge tube with two additional 10-ml portions of phosphate-buffered saline, and the cells were sedimented by centrifuging for 5 min at 300 x g at room temperature. All further steps were carried out at 4°. After removal of the supernatant solution by aspiration, the cell pellet was dispersed in 5 ml of Tris-KCl buffer (0.15 M KCl; 0.01 M Tris-HCl, pH 7.5; and 0.05% (v/v) mercaptoethanol) and was transferred to a tared graduated conical plastic centrifuge tube with another 5 ml of buffer. The suspension was then centrifuged for 10 min at 270 x g; the supernatant solution was removed as completely as possible; and the cell pellet was weighed. Confluent cultures from 10 T-250 flasks usually gave a cell pellet of 500 to 600 mg. The cells were next redispersed in Tris-KCl buffer, and the volume of the resulting suspension was adjusted to 4.0 ml. At this point, a drop of cell suspension was examined for plasma membrane integrity by means of trypan blue dye exclusion: usually fewer than 10% of the cells became stained. The cells were broken by treatment with a 15-s burst of 20 ke x⁻¹ ultrasound at a peak-to-peak amplitude of 5 µm in an MSE 100 watt ultrasonic disintegrator (Measuring & Scientific Equipment Ltd., London, England) at 0°. All cells were disrupted as verified by phase-contrast microscopy. The suspension of disrupted cells was next centrifuged at 100,000 x g for 60 min. The portion of the resulting particle-free supernatant solution lying below the superficial lipid layer was removed with a Pasteur pipette. These particle-free protein solutions from all experimental groups were assayed for phenylalanine hydroxylase activity within 2 to 3 hours of this step; their protein content was measured by the method of Lowry et al. (9) with bovine serum albumin as a standard.

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and homogenized in 3.5 ml of Tris-KCl buffer with a motor-driven Potter-Elvehjem type smooth walled homogenizer having a tolerance of 0.10 to 0.15 mm. Homogenization routinely consisted of 8 complete up-and-down strokes of 5 to 7 s duration each and was carried out with constant cooling in an ice bath. The homogenate was then transferred to a graduated conical plastic centrifuge tube, and its volume was adjusted to 4.0 ml.

For all subsequent steps, including ultrasonic disruption, the rat liver homogenate was treated as the suspensions of cultured cells.

**Assay of Phenylalanine Hydroxylase**—Phenylalanine hydroxylase was assayed by the spectrophotometric method of Ayling et al. (10) in which the phenylalanine-dependent oxidation of a tetrahydropteridine (6,7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (11)) is measured by taking the difference of the absorbance of the dihydro- and tetrahydropteridine at 330 nm.

In the assays (final volume 1.0 ml), the sample cuvette contained 0.1 ml of 1 M Tris-HCl buffer, pH 7.4; 0.05 ml of a 20 mM phenylalanine solution; the volume of the protein solution to be assayed; and sufficient distilled water to make 0.90 ml. The reference cuvette contained all of these components except phenylalanine. After a 5- to 10-min equilibration at 27°, the reaction was started by the simultaneous addition of 0.1 ml of a 1.7 mM solution of 6,7-dimethyl-2-amino-4-hydroxytetrahydropteridine hydrochloride to the sample and reference cuvette. Light absorbance was measured in a Unicam model SP-1800 double-beam recording spectrophotometer. The stoichiometry of the phenylalanine hydroxylation reaction under the conditions of this assay has been demonstrated with extracts of cultured cells as well as with preparations from various animal tissues to be 1 mole of tyrosine formed per mole of enzymatically oxidized tetrahydropteridine (cf. Table III). For determination of tyrosine the spectrophotometric reactions were terminated by the rapid addition of 0.25 ml of 30% trichloroacetic acid solution to the cuvettes. Tyrosine was then determined in the deproteinized samples spectrofluorometrically with a Farrand Spectrofluorometer (Mark I; Farrand Optical Co., Inc., New York) by the methods of Udenfriend and Cooper (11a) and Waalkes and Udenfriend (11b). Standard curves for the tyrosine determinations were prepared by the inclusion of 2 to 30 nmole of tyrosine, in addition to the usual amount of phenylalanine and pteridine cofactor, in incubation mixtures in which the enzyme was first killed by the addition of trichloroacetic acid. One enzyme unit is defined as the phenylalanine-dependent oxidation of 1 nmole of tetrahydropteridine to the dihydro form, or the formation of 1 nmole of tyrosine per min. Under the conditions of the spectrophotometric assay and with the amounts of protein used, substitution of either tyrosine or tryptophan (1 mm) in place of phenylalanine produced no detectable reaction either in the extracts of cultured cells or of liver, except in one cell extract which gave a very slight reaction with tyrosine.

Except when high initial velocities were recorded, the phenylalanine hydroxylase reaction rates of cell extracts were linear with respect to time for several minutes. For rat liver and cell extracts the initial reaction rate was linear with respect to the amount of extract assayed only up to 0.6 to 0.7 mg of protein (Fig. 1). The extracts were routinely assayed at several concentrations of protein and all calculations of phenylalanine hydroxylase-specific activities and total enzyme levels were based on only those initial reaction rates that were proportional to the amount of protein assayed.

**Effect of Cycloheximide on the Incorporation of [G-3H]Phenylalanine into Cellular Proteins**—All experimental media contain-

![Fig. 1. Initial reaction rates of phenylalanine hydroxylase in extracts of H4-II-E-C3 cells and adult rat liver as a function of the amount of protein assayed. Thirty replicate flasks of H4-II-C3 cells were cultivated to confluency in 5F, 20F(S-77, 2G) medium. Eighteen hours before harvesting, one half of the cultures were given fresh 5F, 20F(S-77, 2G) medium alone, while the other half were given the same medium supplemented with 10 mCi hydrocortisone sodium succinate. The cells from the 15 flasks in each experimental group were harvested and pooled. Extracts from the cultured cells and from 1.5 g of rat liver were prepared as described in the text in 6.0 ml of Tris-KCl buffer, A, 0.06 ml (0.28 mg of protein) to 0.75 ml (3.5 mg of protein) of extract from control cells (●) and 0.06 ml (0.34 mg of protein) to 0.75 ml (4.3 mg of protein) of extract from hydrocortisone-treated cells (○) were assayed. B, 0.01 ml (0.29 mg of protein) to 0.32 ml (0.93 mg of protein) of rat liver extract were assayed in duplicate; individual values are shown.](https://example.com/fig1.png)
the rat liver enzyme, when assayed with 6,7-dimethyltetrahydropterin was only 0.17 mM. The \( K_m \) value for phenylalanine with us are not maxima because the concentration of phenylalanine by us are not maxima because the concentration of phenylalanine in the assays was only 1 mM and that of the 6,7-dimethyl-2-amino-4-hydroxytetrahydropteridine hydrochloride were from Calbiochem, San Diego, California; tris-hydroxymethylaminomethane base, sodium hydrocortisone 21-succinate, corticosterone, and cycloheximide were from Sigma Chemical Company, St. Louis, Missouri; the buffer salts and Triton X-100 were from J. T. Baker Chemical Company, Phillipsburg, N. J.; fetal bovine, calf, and horse sera were from Grand Island Biological Company and Gray Industries, Ft. Lauderdale, Florida; \( N^6, \text{O}^2'-\text{dibutyryl} \) cyclic adenosine 3',5'-monophosphosphate (dibutyryl cyclic-AMP) was from Sigma Chemical Company and Calbiochem; trypsin (1:300) was from Nutritional Biochemicals, Cleveland, Ohio; bovine serum albumin (Pentex Fraction V, fatty acid free) was from Miles Laboratories Inc., Kankakee, Illinois; and \( [\text{G-3H}] \) phenylalanine (specific activity, 406 Ci per mole) was from Amersham-Searle Corporation, Arlington Heights, Illinois. The dexamethasone sodium phosphate was the gift Dr. Lazaro E. Gerschenson. The adult male Sprague-Dawley rats were provided by Dr. Sidney Roberts, Department of Biological Chemistry, UCLA School of Medicine, from his own breeding colony.

RESULTS

Comparison between Phenylalanine Hydroxylase Activity of Confluent H4-II-E-C3 Cells after Continuous Cultivation on 5F, 5C(S-77,2G) Medium and that of Adult Rat Liver—The activity of phenylalanine hydroxylase was calculated from measurements of initial reaction velocities with the high speed supernatant fractions of either disrupted cells or rat liver homogenates. Since the activities were normalized per mg of soluble protein in the extracts, it was recognized that any factor that either altered the initial enzymatic rates of extracts or changed their soluble protein contents would necessarily affect the calculated values for specific activity. Because the cultured cells were routinely disrupted by sonication, the effect of ultrasound on the yield of phenylalanine hydroxylase and soluble protein from rat liver homogenates was first determined. Sonication, applied after homogenizing the liver, neither increased nor decreased the total enzyme units in the soluble supernatant, but it consistently increased the concentration of the soluble protein in the extract. Therefore, in all experiments in which the phenylalanine hydroxylase activities of H4 cell and rat liver extracts were compared, the rat liver homogenates were sonicated in parallel along with the cell suspensions. Total enzyme yields per g wet weight of packed cells or tissue were calculated from the measured values for the initial reaction rates, soluble protein concentrations, and sample wet weights. Table I gives the mean values for the phenylalanine hydroxylase specific activities and total enzyme contents of confluent H4 cells cultured on 5F, 5C(S-77,2G) medium and adult rat liver, calculated from the data pooled from several experiments.

\(^{1}\)The values of phenylalanine hydroxylase activities measured by us are not maxima because the concentration of phenylalanine in the assays was only 1 mM and that of the 6,7-dimethyltetrahydropterin was 0.17 mM. The \( K_m \) value for phenylalanine with the rat liver enzyme, when assayed with 6,7-dimethyltetrahydropterin, has been reported to range from 0.76 mM to 1.25 mM (10, 14, 15). The \( K_m \) value for the 6,7-dimethyltetrahydropterin, measured in our laboratory (10), is 0.09 ± 0.007 mM. Nevertheless, the levels of phenylalanine hydroxylase assayed by us in rat liver (900 ± 100 units per g) compare well with the values determined by McGee et al. (14) by two different methods: 970 ± 77 and 1120 ± 122 units per g.

\(^{5}\)Whereas the specific activity of the cell extracts was usually about one-third of that of the rat liver extracts, the total enzyme content per g wet weight of the cells was only about one-tenth of that of liver. This discrepancy results from the fact that the soluble (and total) protein content of rat liver is much greater than that of the cells.

Effect of Adrenocorticosteroid Hormones on Phenylalanine Hydroxylase Activity of H4-II-E-C3 Cells—When H4 cells were cultivated continuously on 5F, 5C(S-77,2G) medium and confluent, slowly replicating populations were placed on either of these media supplemented with \( 10^{-5} \) M hydrocortisone, and in the stationary phase, stimulation of cellular phenylalanine hydroxylase activity over basal enzyme levels was obtained. Moreover, if confluent cells, cultured continuously on 5F, 5C(S-77,2G) medium, were washed free of serum-containing medium and incubated for 16 to 20 hours on serumless S-77,2G medium supplemented with \( 10^{-5} \) M hydrocortisone, the hormone evoked a 4- to 5-fold stimulation of cellular phenylalanine hydroxylase activity over basal enzyme levels in the absence of serum (Table II, see also Fig. 1). This apparent enhancement of the hydrocortisone effect was caused by decreased basal enzyme activities and not by increased cellular phenylalanine hydroxylase levels in the presence of the hormone since hormone-stimulated enzyme activities under these conditions were equal to or even somewhat lower than the phenylalanine hydroxylase levels evoked by the steroid in serum-containing medium. Cultivation of the cells on 5F, 5C(S-77,2G) medium supplemented with \( 10^{-5} \) M sodium succinate under the same conditions produced no stimulation of cellular enzyme activity, nor did the addition of hydrocortisone to the enzyme assay mixture in vitro increase the initial reaction rates of extracts from basal cells.

In order to ascertain that the increased reaction rates seen in extracts of hydrocortisone-treated cells resulted from a true increase of enzyme activity and were not artifacts, the amount of tyrosine formed was determined at the termination of the spectrophotometric assay as described under “Methods and Materials.” The data of Table III demonstrate the satisfactory stoichiometric agreement between the phenylalanine dependent oxidation of the tetrahydropteridine and the amount of tyrosine formed not only in the rat liver extract, but also in extracts of hydrocortisone-treated cells. The data of eight and nine separate experiments for the cultured cells and for rat liver, respectively.
**TABLE II**

**Effect of hydrocortisone on phenylalanine hydroxylase activity of H4-II-E-C3 cells in presence and absence of serum**

Cells cultivated continuously on 5F, 5C(S-77,2G) medium were inoculated into 40 replicate flasks containing 5F, 5C(S-77,2G) medium, and the cultures were grown to confluency. Fifteen hours before harvesting, the cells in 20 of the flasks were washed twice with 10 ml per flask of serumless S-77, 2G medium, and each decade of flasks was given a medium change with either S-77, 2G medium alone or S-77, 2G medium supplemented with 10⁻⁶ M hydrocortisone. At the same time, each decade of the remaining 20 flasks was given a medium change with either 5F, 5C(S-77,2G) medium alone or 5F, 5C(S-77,2G) medium supplemented with 10⁻⁶ M hydrocortisone. The cultures were harvested and the cell extracts were prepared and assayed for phenylalanine hydroxylase activity as described under “Methods and Materials.”

| Culture medium | Enzyme units/g wet weight of cells |
|----------------|----------------------------------|
| 5F, 5C(S-77,2G): |                                  |
| Without hydrocortisone | 110                               |
| With hydrocortisone | 270                               |
| S-77, 2G: |                                  |
| Without hydrocortisone | 49                                |
| With hydrocortisone | 190                               |

**TABLE III**

**Assay of phenylalanine hydroxylase in extracts of rat liver and cultured H4 hepatoma cells by direct spectrophotometric method and by fluorometric determination of tyrosine formed from phenylalanine**

In Experiment 2, 40 flasks of H4-cells were grown to confluency on 5F, 5C(S-77,2G) medium. Twenty-two hours before harvesting, 20 flasks were given a change of fresh medium and the other 20 received the same medium but supplemented with 10⁻⁵ M hydrocortisone. Cells from groups of 10 flasks were used for preparation of cell extracts (10 flasks each for Control 1 and Control 2, and 10 flasks each for the two hydrocortisone-treated groups).

| Tissue or cell extract | Time of incubation (min) | Protein assay (mg) | DMPH or DNH oxidized | Tyrosine formed | Initial reaction velocity calculated | Total enzyme units per g tissue or cells calculated |
|------------------------|--------------------------|--------------------|----------------------|-----------------|-----------------------------------|--------------------------------------------------|
|                        |                          |                    |                      |                 |                                   |                                                  |
| **Experiment 1**       |                          |                    |                      |                 |                                   |                                                  |
| Rat liver              | 2                        | 0.65               | 12.0                 | 12.8            | 6.0 6.4                           | 1200 1280                                        |
|                        | 3                        | 0.68               | 18.3                 | 17.4            | 6.1 5.8                           | 1230 1160                                        |
|                        | 4                        | 0.68               | 24.0                 | 25.0            | 6.3 6.3                           | 1200 1260                                        |
| **Experiment 2**       |                          |                    |                      |                 |                                   |                                                  |
| H4-cells               | 3                        | 0.93               | 8.79                 | 8.49            | 2.03 2.82                         | 118 113                                         |
| Control 1              | 3                        | 0.98               | 8.76                 | 9.50            | 2.92 2.17                         | 119 129                                         |
| Control 2              | 3                        | 0.95               | 16.22                | 15.80           | 5.41 5.27                         | 230 224                                         |
| HC-treated             | 3                        | 0.93               | 15.70                | 15.20           | 5.33 5.07                         | 230 224                                         |
| HC-treated b           | 3                        | 0.93               | 15.70                | 15.20           | 5.33 5.07                         | 230 224                                         |

- a2-Aminotetrahydroxypyrrolidine.
- b Cells exposed to 10⁻⁶ M hydrocortisone for 22 hours.

control and hydrocortisone-stimulated H4 cells. In all further experiments to be reported the spectrophotometric assay was used exclusively.

Next, the effect of hydrocortisone concentration on the phenylalanine hydroxylase activity of cells cultured either in the presence or in the absence of serum was examined. The dose-response profiles obtained are shown in Fig. 2, A and B. Half-maximal response was seen at about 4 × 10⁻⁴ M hydrocortisone under both culture conditions, and maximal stimulation was obtained at about 5 × 10⁻⁷ M and 1 × 10⁻⁵ M hydrocortisone in the presence and absence of serum, respectively.

The time course of the hydrocortisone-evoked stimulation of phenylalanine hydroxylase activity was also investigated in both
the presence and the absence of serum. As is shown in Fig. 3, the cells responded faster to hydrocortisone in the absence of serum than in its presence.

Dependence of Hydrocortisone Effect on Protein Synthesis—The effects of varying concentrations of cycloheximide on general protein synthesis in H4 cells are illustrated in Fig. 4. In this experiment, the incorporation of [3H]phenylalanine into total trichloroacetic acid-precipitable material was measured over a 1-hour period at 37° in the absence of serum. Although this brief exposure to the drug at a concentration of $10^{-4} \text{M}$ had no effect on subsequent cell viability, a 12-hour incubation with only $10^{-5} \text{M}$ cycloheximide, even in the presence of serum, resulted in pronounced cytotoxic effects. Therefore, since no such cytotoxicity was apparent after as much as a 17-hour exposure to $10^{-5} \text{M}$ cycloheximide—a level of the drug that had been found to inhibit general cellular protein synthesis by 70%—this concentration of the anti-metabolite was chosen for the experiment shown in Fig. 5. In this experiment, the continuous presence of $10^{-5} \text{M}$ cycloheximide over a 17- to 18-hour period in serum-free medium blocked the hydrocortisone effect by 60% to 65%, but did not significantly decrease basal cellular enzyme levels. Furthermore, a 24-hour preincubation with $10^{-6} \text{M}$ hydrocortisone had only a slight effect on general protein synthesis in the cells as judged by an increase of approximately 10% in the incorporation of [3H]phenylalanine into cellular proteins. It is, therefore, concluded (a) that hydrocortisone stimulates phenylalanine hydroxylase activity in H4 cells by a mechanism dependent upon de novo protein synthesis and (b) that the hydrocortisone effect on cellular protein synthesis is specific to a relatively small number of cellular proteins, one of which may well be the phenylalanine hydroxylase enzyme itself.

Effect of Steroid Hormones other than Hydrocortisone and Dibutyryl Cyclic Adenosine 3',5'-Monophosphate on Phenylalanine Hydroxylase Activity of H4-II-E-C3 Cells—As is indicated in Table IV, a 16- to 19-hour exposure to either dexamethasone sodium phosphate or corticosterone at a concentration of $10^{-4} \text{M}$ in the presence of serum produced an elevation of cellular phenylalanine hydroxylase activity essentially equivalent to a maximum stimulation by hydrocortisone under the same conditions. In addition, preliminary experiments have also indicated that cellular phenylalanine hydroxylase levels are altered by the presence of cyclic nucleotides. A 16- to 19-hour culture in medium containing medium in the presence of $10^{-4} \text{M}$ dibutyryl cyclic adenosine 3',5'-monophosphate, but in the absence of methyl xanthines, stimulated phenylalanine hydroxylase activity 1.5- to 2-fold over basal levels. A more thorough examination of the influence of cyclic nucleotides on the regulation of this enzyme in H4 cells is being carried out.

Examination of Additional Cell Lines of Hepatic Origin for Phenylalanine Hydroxylase Activity—Several cell lines of hepatic origin were examined for the presence of phenylalanine hydroxylase activity under conditions essentially identical with those described for the H4 cells. In every instance, extracts from the
proband cells were prepared and compared with extracts of H4 cells and/or of rat liver. With the single exception of the C-15 cells and the four epithelioid subclones derived from them, all cell lines were assayed both under basal conditions and after a 16- to 20-hour exposure to $10^{-8}$ M hydrocortisone. The eight remaining flasks were washed with the serumless medium as described above and a pair of flasks was incubated under each of the experimental conditions except that the medium also contained $2 \times 10^6$ dpm of [G-3H]phenylalanine. Two flasks containing cells killed at 100° were the controls. After 18 hours of incubation the incorporation of 3H into proteins was measured and is expressed (black columns) as a percentage of the specific activity of the proteins found in the pair of flasks in the absence of hormone and cycloheximide (262,400 dpm per mg). Components of incubations in addition to the basal S-77,2G medium are indicated above the columns: AF, SC, 5% fetal bovine and calf sera; CX, cycloheximide; HC, hydrocortisone.

The following cell lines showed no detectable phenylalanine hydroxylase activities when the assays were made on 1.2 to 9.0 mg of protein: the parental clone and four subclones of C-15 cells, derived from normal rabbit liver; HTC cells, originating from a Morris rat hepatoma; and the RLC and RL cells, both derived from normal rabbit liver; HTC cells, originating from a Morris rat hepatoma; and the RLC and RL cells, both derived from normal rabbit liver. The only cell type in which phenylalanine hydroxylase activity was detected in addition to the H4 cells, that the phenylalanine hydroxylase content of the H4 cells under basal conditions is only about 2.1% of that of the liver cells and from the data of Table I, and on the assumption that the levels of phenylalanine hydroxylase found in the cultured cells and/or of rat liver, one needs first to compare the levels of phenylalanine hydroxylase found in the cultured cells with those of the liver in vivo. The phenylalanine hydroxylase activity of H4 cells per g wet weight was about 10% of that of adult rat liver. One gram of packed H4 cells grown under our experimental conditions contains 4.5 $\times 10^9$ cells. According to a recent report 1 cm$^3$ (or $\sim$ g) of adult rat liver contains $1.0 \times 10^9$ parenchymal cells (16). It is calculated from these figures and from the data of Table I, and on the assumption that the enzyme in the liver is localized exclusively in the parenchymal cells, that the phenylalanine hydroxylase content of the H4 cells under basal conditions is only about 2.1% of that of the liver cells ($2.0 \times 10^{-7}$ units versus $96 \times 10^{-7}$ units per cell). However, the difference between the enzyme content of the two cell types becomes much less once the calculations are related to cytoplasmic volume. One of the main morphological characteristics of the H4 cells is their small cytoplasm as compared to the cytoplasm of the liver parenchymal cells; the size of the nuclei of the two cells, on the other hand, is very similar (Fig. 6). We have determined in 10-μm thick sections, made from pellets of H4 cells and adult rat liver fixed in Bouin’s solution and stained with hematoxylin and eosin, the ratio of the volume of cytoplasm to that of the nucleus by the method of Chalkley (17). These measurements showed that the cytoplasmic volume of the H4 cells was only about one-fourth of that of the liver cells. It follows, therefore, that the phenylalanine hydroxylase content of basal

![Figure 5](http://www.jbc.org/)

**Fig. 5.** The effects of cycloheximide on phenylalanine hydroxylase levels and on protein synthesis in presence and absence of hydrocortisone. Fifty-eight replicate flasks of cells were grown to early confluence on 5F,SC(S-77,2G) medium. Eighteen hours before harvesting, 10 flasks were given a change of 5F,SC(S-77,2G) medium. Forty cultures were washed twice with 8 ml of serumless S-77,2G medium and were given 20 ml of S-77,2G medium alone or the same medium supplemented with $10^{-8}$ M hydrocortisone, $10^{-8}$ M cycloheximide, or $10^{-6}$ M hydrocortisone plus $10^{-6}$ M cycloheximide as indicated in the figure. The cells were harvested, and the cell extracts were assayed for phenylalanine hydroxylase. The total enzyme content per g wet weight of packed cells is expressed as a percentage of the value (120 units per g) obtained for the cells incubated for 18 hours in serumless medium in the absence of hydrocortisone and cycloheximide.

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**Table IV**

| Addition                      | Concentration of additions | Total enzyme content (units/g wet weight) |
|-------------------------------|---------------------------|----------------------------------------|
| Experiment 1                  |                           |                                        |
| None                          | 0                         | 100                                    |
| Hydrocortisone                | $10^{-8}$ M               | 250                                    |
| Dexamethasone                 | $10^{-8}$ M               | 210                                    |
| Dibutyryl cyclic AMP          | $10^{-8}$ M               | 220                                    |
| Experiment 2                  |                           |                                        |
| None                          | 0                         | 87                                     |
| Hydrocortisone                | $10^{-8}$ M               | 220                                    |
| Corticosterone                | $10^{-8}$ M               | 180                                    |
| Dibutyryl cyclic AMP          | $10^{-8}$ M               | 120                                    |

**DISCUSSION**

In an evaluation of the validity of the use of these cultured hepatoma cells as an experimental model for studying the regulatory mechanisms governing the expression of the phenylalanine hydroxylase gene in mammalian liver, one needs first to compare the levels of phenylalanine hydroxylase found in the cultured cells with those of the liver in vivo. The phenylalanine hydroxylase activity of H4 cells per g wet weight was about 10% of that of adult rat liver. One gram of packed H4 cells grown under our experimental conditions contains $4.5 \times 10^9$ cells. According to a recent report 1 cm$^3$ (or $\sim$ g) of adult rat liver contains $1.0 \times 10^9$ parenchymal cells (16). It is calculated from these figures and from the data of Table I, and on the assumption that the enzyme in the liver is localized exclusively in the parenchymal cells, that the phenylalanine hydroxylase content of the H4 cells under basal conditions is only about 2.1% of that of the liver cells ($2.0 \times 10^{-7}$ units versus $96 \times 10^{-7}$ units per cell). However, the difference between the enzyme content of the two cell types becomes much less once the calculations are related to cytoplasmic volume. One of the main morphological characteristics of the H4 cells is their small cytoplasm as compared to the cytoplasm of the liver parenchymal cells; the size of the nuclei of the two cells, on the other hand, is very similar (Fig. 6). We have determined in 10-μm thick sections, made from pellets of H4 cells and adult rat liver fixed in Bouin’s solution and stained with hematoxylin and eosin, the ratio of the volume of cytoplasm to that of the nucleus by the method of Chalkley (17). These measurements showed that the cytoplasmic volume of the H4 cells was only about one-fourth of that of the liver cells. It follows, therefore, that the phenylalanine hydroxylase content of basal
stimulated enzyme activities can be augmented by a further factor of 2 when the cells are cultivated continuously in the presence of horse serum under some conditions. The sensitivity of the cells to other hormones and humoral factors has not yet been investigated, nor have steroids and cyclic nucleotides been tested in combination in order to examine additive or possible synergistic effects. It seems, therefore, probable that, by a combination of specific cell-culture media and supplemental hormones, the levels of phenylalanine hydroxylase in the cultured cells, expressed per unit volume of cytoplasm, can be raised to values comparable to those found in the parenchymal cells of rat liver. Current experiments are being directed at a more complete delineation of the various factors necessary to evoke a maximum expression of the phenylalanine hydroxylase gene in these cultured hepatoma cells.

Studies on whole animals and perfused organs have demonstrated that a number of enzymes characteristic of the differentiated liver are under hormonal control in vivo (18). Moreover, the activities of several of these hepatic marker enzymes are influenced specifically by alterations in the levels of circulating adrenocorticosteroids (19-21). In concordance with these studies, the adaptation of differentiated hepatic tissues to the conditions of cell culture has recently yielded clonal cell populations that possess a number of liver-specific functions (1-3, 5, 6, 22-27), many of which still retain much, if not most of their original in vivo responsiveness to hormones and other humoral factors (1-3, 13, 22, 25, 28-34). Nevertheless, in spite of the growing number of these examples, the phenylalanine hydroxylase enzyme has not been reported previously to be present in any permanent cell line of liver origin, and only little evidence has been presented for the regulation of this enzyme by hormones in the whole animal (14, 35, 36). Eagle et al. (37) noted, however, the conversion of phenylalanine into tyrosine in a strain of HeLa cells cultured in the absence of tyrosine, and Cartwright and Danks (38) reported phenylalanine hydroxylase in primary cultures of human fetal muscle fibroblasts.

Our work demonstrates, first, that phenylalanine hydroxylase is present in two established cell lines derived from transplantable rat hepatomas and, second, that the activity of this enzyme is modified in a similar manner in both cell types by physiological concentrations of adrenocorticosteroid hormones. Although the ultimate confirmation that the sensitivity of phenylalanine hydroxylase in the cultured cells to regulation by steroid hormones or other humoral factors is a true representation of the in vivo situation must await further whole-animal and liver-perfusion studies, the initial observation by Freedland (35) of steroid-evoked stimulation of phenylalanine hydroxylase activity in adult rats coupled with the recent report by McGee et al. (14) describing a 2-fold enhancement of hepatic phenylalanine hydroxylase levels after hydrocortisone injection during a brief period in the developing rat would suggest that adrenocorticosteroids probably play an important role in the maintenance of normal levels of this enzyme in vivo. Moreover, by analogy with the tyrosine aminotransferase system in H4 and HTC cells, the sensitivity of which to steroids, cyclic nucleotides, and insulin does substantiate the findings from earlier studies with perfused livers (28-34), it is tempting to speculate that the regulatory mechanisms governing the expression of the phenylalanine hydroxylase gene in these cultured cells also exist in the mammalian liver in vivo.

The similarities between the behavior of the phenylalanine hydroxylase and tyrosine aminotransferase systems of the H4 cells are striking. Reel et al. (31) have reported an 8- to 10-fold

![Fig. 6 Photomicrographs of hematoxylin and eosin-stained sections of rat liver (A) and a pellet of H4-II-E-C3 cells (B) grown on 5F,5C(S-77,2G) medium. × 740. The H4 cells have a much smaller cytoplasmic volume than the liver cells. The cytological appearance of H4 cells grown in the presence of 10⁻³ M hydrocortisone could not be distinguished in such sections from that of cells grown without the hormone.](http://www.jbc.org/)

H4 cells per unit of cytoplasmic volume is about 8.4% of that of the liver parenchymal cell. The steroid hormones, which caused no measurable change in the ratio of cytoplasmic to nuclear volumes, were shown to increase 2- to 2.5-fold the phenylalanine hydroxylase levels of the H4 cells. Thus, adrenocortical steroids can elevate cellular enzyme activities per unit volume of cytoplasm to values that are up to 21% of those of liver parenchymal cells in vivo. Furthermore, recent experiments, to be reported later, on the influence of the nature of the sera used in the culture media on cellular enzyme levels have shown that hydrocortisone-
maximum "induction" of tyrosine aminotransferase activity by hydrocortisone after a 24-hour preincubation of the cells on a medium free of serum and hormone. In our studies, the maximum stimulation of phenylalanine hydroxylase activity obtained with hydrocortisone was 4- to 5-fold on a serumless medium (Table II). However, in our experiments the cultures were placed on the experimental media directly from serum-containing medium without any preincubation in the absence of serum. Under those conditions, basal enzyme activities were seen to decrease to about one-half of their former values by 16 to 20 hours. If this decay of basal phenylalanine hydroxylase activity were to have continued with the same half-life for an additional 24 hours, the degree of stimulation by hydrocortisone would have been at least 8- to 10-fold. We found, in an experiment not reported here, that a 24-hour preincubation of the cells on serumless S-77, 2G medium in the absence of hydrocortisone did not affect the maximum enzyme levels attained upon subsequent incubation with the hormone. Hence, the degree of stimulation of phenylalanine hydroxylase by hydrocortisone appears to be comparable to that of tyrosine aminotransferase in the H4 cells. The time course of the effect of hydrocortisone on these two enzymes is also similar, with maximum stimulation of activity occurring by 12 hours in absence of serum in both systems (Reference 31 and Fig. 3B). However, the response of the phenylalanine hydroxylase and tyrosine aminotransferase enzymes of H4 cells to varying dosages of hydrocortisone in the absence of serum was somewhat different. The stimulation of tyrosine aminotransferase took place over a broader range of hydrocortisone concentrations (1 X 10^{-9} M to 5 X 10^{-7} M) (28) than did that of phenylalanine hydroxylase (1 X 10^{-10} M to 1 X 10^{-7} M) (Fig. 2B), and, in addition, Barnett and Wicks (32) observed that concentrations of dexamethasone (and hydrocortisone) greater than 10^{-4} M "produced variable and sometimes inhibitory effects" on the induction of the aminotransferase. In contrast to our findings with phenylalanine hydroxylase (Table II), basal tyrosine aminotransferase activity appears to be stable in the absence of serum (34, 39), whereas, at least in HTC (2) and RLC (3) cells, hydrocortisone stimulates the phenylalanine hydroxylase activity of H4 cells to varying degrees of hydrocortisone-stimulated phenylalanine hydroxylase activity of H4 cells has not yet been investigated.

In a manner analogous to the tyrosine aminotransferase system of HTC (2) and RLC (3) cells, hydrocortisone stimulates the phenylalanine hydroxylase activity of H4 cells by a mechanism dependent upon protein synthesis (Fig. 5B). In two experiments, hydrocortisone had only a slight effect, if any, on general protein synthesis in the cells, as judged by incorporation of[^{3}H]phenylalanine into protein, and the addition of the hormone directly to cell extracts in vitro did not enhance the phenylalanine hydroxylase activity. The simplest interpretation of these observations is that hydrocortisone stimulates cellular enzyme activities by inducing the synthesis of new phenylalanine hydroxylase protein; however, the alternative possibility that the hormone induces the synthesis of a second enzyme which, in turn, activates phenylalanine hydroxylase cannot be ruled out.

Acknowledgments—We are grateful to Dr. Van R. Potter, McArdle Laboratory, University of Wisconsin, Madison, Wisconsin; to Dr. E. Brad Thompson, National Cancer Institute; National Institutes of Health, Bethesda, Maryland, and to Dr. Lazaro E. Gerschenson, Laboratory of Nuclear Medicine and Radiation Biology, University of California Los Angeles, for generously giving us initial cell cultures. We also thank our colleague, Dr. June Ayling, for permitting us to use her method of phenylalanine hydroxylase assay before its publication.

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