Effect of Serum on Phenylalanine Hydroxylase Levels in Cultured Hepatoma Cells*

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Continued high levels of phenylalanine hydroxylase in cultured H4 II E C3 rat hepatoma cells require either serum or glucocorticoids in the culture medium. Upon withdrawal of serum, cellular phenylalanine hydroxylase levels decay exponentially with a half-life of 22 hours for about 60 hours, after which time a low, constant enzyme content persists for at least 96 hours. This decline of phenylalanine hydroxylase is fully reversible; normal enzyme levels are restored in a time- and dosage-dependent fashion upon addition of serum to basal cultures. The serum factor is nondialyzable and moderately heat-stable. The stimulation by serum of the phenylalanine hydroxylase content of basal cultures is blocked by 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide and requires ongoing cellular protein synthesis. When added to the enzyme-assay mixture in vitro, serum does not alter the phenylalanine hydroxylase activity of extracts from basal cultures. Three lines of evidence suggest that serum contains a nonsteroidal phenylalanine hydroxylase stimulatory component(s): (a) glucocorticoid antagonists inhibit less than one-half of the biological activity of serum; (b) exhaustive extraction of endogenous serum glucocorticoids with charcoal reduces the activity of serum to about one-half of control values; and (c) the stimulatory effects of charcoal-extracted serum and hydrocortisone are additive. The phenylalanine hydroxylase stimulatory activities of the charcoal-extracted sera from four mammalian species and from three stages in development in one mammalian species are comparable. A survey of partially purified preparations of a number of known hormones failed to reveal any one capable of elevating the phenylalanine hydroxylase levels of basal cultures in a manner comparable to that of charcoal-extracted serum.

Phenylalanine hydroxylase, found in only a few mammalian tissues (liver (1-4), kidney (1-4), and pancreas (1)), is thought to be a differentiated function characteristic of those tissues. With the aim of finding a model system for the study of the mechanisms regulating the expression of the phenylalanine hydroxylase gene in differentiated tissues, a number of cell culture lines of hepatic origin were surveyed for phenylalanine hydroxylase. This enzyme was found, so far, in only two established cell lines (H4-II-E-C3 and MH,C,), each derived from a minimum deviation rat hepatoma (5, 6), among over a dozen studied (7). With the first of these lines, it was observed that withdrawal of serum from the cultures resulted in a decline in their phenylalanine hydroxylase content and that hydrocortisone, when added to serum-free or serum-containing medium, elevated cellular enzyme levels (7). This stimulation by hydrocortisone depended on protein synthesis, as the action of hydrocortisone was inhibited by cycloheximide (7).

The responses of phenylalanine hydroxylase in the H4 and MH,C, cells to serum and glucocorticoids (7) are similar, but the H4 cells are more suitable for experimentation than the MH,C, cells because they have a shorter doubling time (about 24 hours) and are more readily detached from the substratum than the MH,C, cells. For this reason, the H4 cells were used for all further experiments.

We report now on the effects of serum and of several hormones on the phenylalanine hydroxylase content of H4 cells. It will be shown that, apart from glucocorticoids and insulin, the serum contains an as yet unidentified component that raises the phenylalanine hydroxylase content of the H4 cells and that this effect, like that of glucocorticoids, requires active protein synthesis.

EXPERIMENTAL PROCEDURE

Cell Culture—The growth of stock cultures of the H4 cells and their subcultivation were carried out as described previously (7) except that the standard growth medium consisted of a modified Swim's Medium S-77 supplemented with 5% (v/v) fetal bovine serum plus 10% (v/v)

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The abbreviations and trivial names used are: hydrocortisone, sodium 17α-hydroxycorticosterone 21-succinate; cycloheximide, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide; H4, H4-II-E-C3 rat hepatoma cells; cycloleucine, 1-aminocyclopentane-1-carboxylic acid; testosterone, androst-4-ene-17α-ol-3-one; fluoxymesterone, 3α-fluoro-11β-hydroxy-17α-methyl testosterone.

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horse serum (8). After detachment of cells from stock cultures with trypsin and determination of cell number by hemocytometry (8), cultures were plated and grown for experiments as follows. Replicate cultures were initiated in 75-cm² flasks by the inoculation of 0.5 to 4.0 × 10⁶ cells into 20 ml of growth medium/flask; the cultures were grown for from 5 to 12 days until confluency was attained and exceeded by 1 or more days (i.e. the cultures were "postconfluent"). It has been shown previously (8) that the phenylalanine hydroxylase levels of the H4 cells become highly stimulated to stimulation by serum or by serum plus added hydrocortisone upon attaining confluency and that 24 hours after confluency the enzyme content of the cells reaches a new high plateau.

Postconfluent cultures were transferred to serum-free medium as follows. Each culture was washed twice with 10 ml of modified Swim’s Medium S-77 (without serum supplement) and was then provided with 20 ml of the serum-free medium. On the 3rd or 4th day after transfer to serum-free medium, the experimental media described in the legends to tables and figures were added. All media were renewed at least every 2 to 3 days until the time of harvesting.

The exposure of postconfluent cultures to serum-free medium for 3 days or longer results in a decay of their phenylalanine hydroxylase content to a low, constant level. Such cultures will be referred to as "postconfluent" and were used for most of the experiments. Deviations from the protocol described above will be noted in the legends to tables and figures.

Preparation of Experimental Media—The partially purified pituitary hormone preparations were dissolved and serially diluted in either distilled water (rat somatotropin) or in 0.9% NaCl solution adjusted to the optimal pH for solubility as recommended by the National Institutes of Health Hormone Distribution Program. The vehicles used for the dissolution of the other nonsteroidal hormones were as follows: epinephrine, distilled water; triiodothyronine, 10 mM KOH; glucagon, 10 mM HCl; and insulin, sterile 4 mM HCl. The vehicle used for the guinea pig anti-insulin serum was sterile phosphate-buffered saline, pH 7.4 (7), containing 50 mM ethylene glycol tetramethyletheracetate. Experimental media were either sterilized after preparation by passage through filters containing cellulose membranes of 0.22 µm pore size (Sterilf, Millipore, XX1104700, or those cited earlier (7)) or prepared by the aseptic addition of small volumes of filter-sterilized (Millex, Millipore, SGLS-02505) serum or concentrated solutions of drugs, steroids, or hormones to measured volumes of sterile medium. When steroids dissolved in ethanol were added to the cultures, the final alcohol concentration in the medium was 0.04% (v/v). When hormones were added to the media in small volumes of aqueous vehicle (at 1 to 5% of the final medium volume), the control medium was supplemented with an equal volume of the vehicle. In the preparation of media containing insulin, filter sterilization was avoided, as insulin had previously been shown to bind to these membranes (8); and 0.1 mg/ml of bovine serum albumin was included both in the final insulin-supplemented medium and in the control medium (cf. Ref. 19).

The section containing the anti-insulin serum was not filter-sterilized either.

Preparation of Cell Extracts and Assay of Phenylalanine Hydroxylase—Cultures were harvested with trypsin (7), and extracts of cells from 2 to 6 culture flasks were prepared by the disruption of 175 to 200 mg of packed cells/ml of buffer containing Triton X-100 detergent as described (8). The particle-free extracts were assayed for phenylalanine hydroxylase activity by the method of Aylng et al. (8, 10); 1 enzyme unit is defined as the phenylalanine-dependent oxidation of 1 nmol of L-phenylalanine to L-tyrosine per min at 37° (7). The reproducibility of the enzyme assay was monitored on the day of each determination by the use of thawed samples of a rat liver extract of known phenylalanine hydroxylase activity that had been stored frozen in liquid nitrogen (8).

Determination of Incorporation of Radioactive Amino Acids into Cellular Components—Method A, for analysis of trichloroacetic acid-insoluble material; this method was performed as described (7) except that the cells were detached from the substratum by treatment with trypsin. Method B, for analysis of trichloroacetic acid-soluble and insoluble material; in order to measure simultaneously the incorporation of radioactive into trichloroacetic acid-soluble and -insoluble intracellular pools, cultures, washed five times with serum-free medium, the experimental media described in the legends to tables and figures.

Results

Effect of Cultivation of Cells in Serum-free Medium on Their Phenylalanine Hydroxylase Content—When confluent cultures of H4 cells were transferred to serum-free medium for 16 to 20 hours, their phenylalanine hydroxylase levels decreased to about one-half of those of replicate cultures maintained in serum-containing medium (7). This decline in cellular enzyme content in serum-free medium continues at an exponential rate for about 60 hours (Fig. 1), after which time a low and constant phenylalanine hydroxylase level persists for up to 96 hours. The half-life of the initial decay is about 22 hours.

That this decrease in the phenylalanine hydroxylase content of cultures is not the result of cell degeneration was substantiated by the following observations. (a) The appearance of these cultures under a phase-contrast microscope gave no indication of cytopathic effects. (b) Cells cultivated for 5 days in serum-free medium resumed growth when provided with...
serum; and when the serum-deprived and serum-supplemented cultures were harvested 3 days later, over 90% of the cells obtained under both conditions excluded trypan blue. (c) The rate of general protein synthesis in serum-deprived and serum-supplemented cultures were equivalent (cf. Table V and "Discussion"). (d) The high phenylalanine hydroxylase content of the cells was restored upon transfer of the cultures from serum-free to serum-containing medium (Figs. 1 and 2).

Since phenylalanine hydroxylase is an iron- and copper-containing enzyme (14, 15), the decline in enzyme activity seen in serum-deprived cultures might have resulted from the deficiency of a trace metal, not present in unsupplemented S-77 medium (16), that had been provided by the serum fraction of the growth medium. Fig. 1, however, also shows that when serum-free S-77 medium was supplemented with levels of Fe++, Cu++, and Zn++ comparable to those present in Ham's Medium F-12 (3 μM, 10 nM, and 3 μM, respectively; see Reference 17), this enriched medium was incapable of preventing the decay of cellular phenylalanine hydroxylase content that occurred in the absence of serum.

Generalized nutritional "step-down" has been shown to increase the rate of degradation of tyrosine aminotransferase in hepatoma tissue culture cells (18). Therefore, in order to rule out the possibility that serum-deprived cultures of H4 cells were incapable of maximal rates of glucose uptake in the absence of insulin, and were accordingly depleted of a source of carbon or high energy metabolites, serum-free S-77 medium was supplemented with either 110 mg/liter of pyruvate (17) or with 400 microunits/ml of insulin (19). These supplemented S-77 media also failed to prevent the decline of cellular phenylalanine hydroxylase content seen in serum-free medium.

All these observations suggested that serum contained a factor(s) needed for the maximum expression of the phenylalanine hydroxylase gene and that the low, but consistent, levels of phenylalanine hydroxylase measured in H4 cells after 3 to 7 days of maintenance in serum-free medium probably represented the constitutive levels of the enzyme in these cells.

**Stimulation by Serum of Phenylalanine Hydroxylase Content of Basal Cultures**—When serum-containing medium was added to basal cultures, cellular phenylalanine hydroxylase levels increased in a time- and dosage-dependent fashion. Fig. 2 depicts the time course of this stimulation of enzyme content by serum and summarizes the data from two separate experi-

![Graph](http://www.jbc.org/)
ments. In the first experiment (Curve A), basal cultures were exposed to the serum complement of the regular growth medium, 5% (v/v) fetal bovine serum plus 10% (v/v) horse serum; in the second experiment (Curve B) they were exposed to 20% (v/v) charcoal-extracted horse serum (equivalent to 15% (v/v) native horse serum; cf., “Experimental Procedure”). The kinetics of the stimulation by the native sera and by the charcoal-extracted horse serum were comparable except that the rate of increase in enzyme levels elicited by the native sera was somewhat greater than that seen with the charcoal-extracted serum. The first detectable elevation of cellular phenylalanine hydroxylase content was seen by 14 hours, with the half-maximal value being reached by 20 hours, with the native sera; while the initial increase in enzyme content was evident by 8 hours, with the half-maximal value occurring at about 22 hours, with the charcoal-extracted horse serum. The effects of these sera were essentially complete by 48 hours, with the maximum phenylalanine hydroxylase content obtained with the native sera and with the charcoal-extracted serum being 290 and 230 units/g, respectively (not shown).

Fig. 3 shows the dosage profile of the effect of native horse serum on the phenylalanine hydroxylase content of basal cultures. The figure summarizes the data from two separate experiments in which the cells were exposed to various concentrations of horse serum (circles) or fetal bovine serum (triangles) for 46 hours (closed symbols) or for 70 hours (open symbols). A detectable elevation of enzyme content was seen with as little as 1% horse serum, and maximum enzyme levels were obtained with concentrations of horse serum equal to or greater than 15%. Heating the horse serum at 65°C for 15 min had no effect on its phenylalanine hydroxylase stimulatory activity (square), nor did four successive dialyses against 100 volumes of 0.9% NaCl solution for 72 hours.

Fig. 3 also shows that the phenylalanine hydroxylase stimulatory activity of native fetal bovine serum was only 30 to 45% of that of native horse serum (but cf. Table II) when tested in parallel on basal cultures at a concentration of 15%. Consequently, of the two sera present in the standard growth medium, horse serum was chosen in all subsequent experiments for the characterization of the active serum component(s).

Evidence for Presence of Nonsteroidal Stimulatory Factor(s) in Serum—Since native mammalian sera might be expected to contain glucocorticoids that could evoke stimulation of phenylalanine hydroxylase levels in the cultured cells (7), next a quantitative estimate of the contribution of endogenous steroid hormones to the serum effect was obtained. Three experimental approaches were taken for this purpose. First, steroid hormones and analogs known to antagonize the action of glucocorticoids (“anti-inducer” steroids, testosterone and fluoxymesterone (20)) were tested for their influence on the stimulatory activity of hydrocortisone and serum. Second, the effect of exhaustive charcoal extraction of serum on its phenylalanine hydroxylase stimulatory capacity was determined. Third, cultures were exposed to maximally stimulatory concentrations of glucocorticoid-free serum and hydrocortisone both separately and in combination to ascertain whether or not additive effects could be observed.

As was demonstrated for the antagonistic effect of testoster-
one and fluoxymesterone in the induction of tyrosine amino-

transferase by glucocorticoids (20), these two anti-inducers inhibited the stimulation of phenylalanine hydroxylase by hydrocortisone. Fluoxymesterone was more potent in this respect than was testosterone. Thus, for example, 10^-4 M fluoxymesterone caused a 79 to 87% inhibition of the stimulation of phenylalanine hydroxylase by 7.5 x 10^-8 M hydrocorti-
sone when the latter was added to cultures in S-77 medium either with or without 5% fetal bovine plus 10% horse serum. Testosterone, at a concentration of 10^-7 M, inhibited the action of hydrocortisone in serum-free medium by only about 30%. That only a part of the stimulatory effect of sera on the phenylalanine hydroxylase content of basal cultures was attributable to their glucocorticoid content was suggested by experiments in which fluoxymesterone and testosterone (10^-5 M each) inhibited the induction of the hydroxylase in basal cultures by the usual serum supplement by only 43% and 17%, respectively. In contrast, the stimulatory effect of charcoal-extracted horse serum (glucocorticoid content <2.7 x 10^-9 M), which retained approximately one-half of the activity of the native serum (see further on), was not influenced at all by 10^-5 M fluoxymesterone; the enzyme contents measured after a 45-hour exposure of replicate basal cultures to 20% charcoal-extracted serum with and without fluoxymesterone were 137 ± 10 and 138 ± 5 units/g, respectively. The experiments with fluoxymesterone suggested that approximately one-half of the stimulatory effect of sera might be attributable to their glucocorticoid content. This estimate is in satisfactory agree-

ment with the results obtained from experiments made by the use of charcoal-extracted serum.

In the experiment shown in Fig. 4, the effect of charcoal extraction on the phenylalanine hydroxylase stimulatory activity of horse serum was determined with basal cell cultures that had been preincubated for 3 days in serum-free medium. In order to monitor the efficacy of the extraction procedure in this experiment, underivatized hydrocortisone was added to a sample of serum in an amount sufficient to produce a final concentration of about 10 µM in the experimental media (see the legend to Fig. 4). One-half of this serum, containing the added steroid hormone, was extracted in parallel with the test serum, while the other half was merely heated along with the control serum (cf., “Experimental Procedure”). A preliminary experiment had indicated that a single extraction with charcoal reduced the stimulatory activity of the test serum by about 40%, but decreased the biological activity of the steroid-supplemented serum only marginally (by about 16%). However, in the experiment shown (Fig. 4), three such extractions lowered the activity of the fortified serum to a value equivalent to that of the test serum. It was further shown in a subse-
quent experiment that the total phenylalanine hydroxylase stimulatory activity of test serum to which hydrocortisone had been added after charcoal extraction was as great as that of hydrocortisone-supplemented, but unextracted serum (also cf., Table I). We conclude, therefore, from these experiments and from those made with the anti-inducer steroids, that endogenous glucocorticoids are responsible for about one-half of the phenylalanine hydroxylase stimulatory activity of native horse serum. Thus, the nonsteroidal component(s) of horse serum alone evokes about a 10-fold increase in the enzyme content of basal cultures under the conditions of these experiments.

The third line of evidence indicating the presence of a nonglucocorticoid phenylalanine hydroxylase stimulatory fac-

tor in serum was provided by experiments in which the
interaction between hydrocortisone and charcoal-extracted serum was examined. In Experiment 1 of Table I, maximally stimulatory concentrations of hydrocortisone and charcoal-extracted serum in combination elevated the phenylalanine hydroxylase levels of basal cultures by an amount about equal to that calculated on the basis of numerical additivity. However, in Experiment 2 of Table I, although the effect of hydrocortisone alone was not as pronounced as in Experiment 1, the effect of either native or charcoal-extracted serum in combination with hydrocortisone was equivalent in magnitude to the stimulation obtained with charcoal-extracted serum in combination with hydrocortisone. Therefore, we conclude that (a) native serum contains a nonsteroidal phenylalanine hydroxylase stimulatory component(s) responsible for about one-half of its stimulatory activity as assayed with basal cultures.

### Table I

| Experiment | Hydrocortisone | Native Serum | Extracted Serum | Phenylalanine Hydroxylase Content |
|------------|----------------|--------------|-----------------|----------------------------------|
| 1          | -              | -            | -               | 31                               |
|            | +              | -            | -               | 590 ± 30                         |
|            | +              | +            | -               | 285 ± 5                          |
|            | +              | +            | +               | 1095 ± 5                         |
|            | +              | -            | +               | 790 ± 30 (875)*                  |
| 2          | +              | -            | -               | 385 ± 5                          |
|            | +              | +            | -               | 350 ± 20                         |
|            | +              | +            | +               | 1145 ± 15                        |
|            | +              | -            | +               | 1065 ± 45 (755)*                 |
| 3          | -              | +            | -               | 28                               |
|            | +              | +            | -               | 1190 ± 20                        |
|            | +              | +            | +               | 1190 ± 9                         |

* Equivalent to 15% native serum; cf. “Experimental Procedure.”

From the preceding experiments we conclude that (a) native serum contains a nonsteroidal phenylalanine hydroxylase stimulatory component(s) responsible for about one-half of its biological activity and (b) both glucocorticoids and this component(s) of serum are required for the maximum expression of the phenylalanine hydroxylase gene in the cultured cells.

### Comparison of Phenylalanine Hydroxylase Stimulatory Activities of Several Mammalian Sera after Extraction with Charcoal

The data from the experiments of Fig. 3 had indicated that native horse serum stimulated phenylalanine hydroxylase in the cells more than did native fetal bovine serum. Because this difference could have resulted from differing concentrations of endogenous glucocorticoids in the two sera, and not from a difference in the contents of their nonsteroidal phenylalanine hydroxylase stimulatory components, biological activities of several mammalian sera were surveyed after extraction with charcoal. Table II summarizes the results obtained.

We conclude from these data that the nonsteroidal phenylalanine hydroxylase stimulatory activities of porcine, bovine, calf, fetal bovine, and human sera are comparable to that of...
horse and that the difference in the activities of native horse and fetal bovine sera observed earlier was attributable to a different content of glucocorticoids in the sera.

**Examination of Known Hormones for Their Ability to Mimic Phenylalanine Hydroxylase Stimulatory Effect of Charcoal-extracted Serum**—In order to examine the possibility that the nonsteroidal component(s) of serum acting on cellular phenylalanine hydroxylase might be any one of a number of already known hormones, various hormones were tested for their ability to stimulate the phenylalanine hydroxylase levels of basal cultures. The following peptide hormones, each tested at concentrations of 0.1, 1.0, and 10 pg/ml, had no effect on the known hormones, various hormones were tested for their

| Extracted serum | Phenylalanine hydroxylase content units/lg |
|-----------------|------------------------------------------|
| Equine          | 170 ± 10                                  |
| Porcine         | 175 ± 5                                   |
| Human           | 160 ± 10                                  |
| Bovine          | 190 ± 10                                  |
| Calf            | 157 ± 23                                  |
| Fetal bovine    | 180 ± 0                                   |

After a 4-day preincubation in serum-free medium, replicate post-confluent cultures received S-77 medium supplemented with the indicated charcoal-extracted sera (cf. "Experimental Procedure") at a volume concentration of 20%. Ninety-one hours later, the cultures were harvested and assayed for phenylalanine hydroxylase. The data shown represent the mean values and deviations from the mean obtained with duplicate determinations under each experimental condition.

**Table II**

*Phenylalanine hydroxylase stimulatory activities of mammalian sera after extraction with charcoal to remove endogenous glucocorticoids*

After a 4-day preincubation in serum-free medium, replicate post-confluent cultures received S-77 medium supplemented with either 20% (v/v) charcoal-extracted serum or the designated hormone at the final concentration indicated in the table. Forty hours later, the cultures were harvested and assayed for phenylalanine hydroxylase.

**Table III**

*Effects of glucagon, insulin, and charcoal-extracted serum on phenylalanine hydroxylase content of basal cultures*

After a 4-day preincubation in serum-free medium, replicate post-confluent cultures were given S-77 medium supplemented with either 20% (v/v) charcoal-extracted serum or the designated hormone at the final concentration indicated in the table. Forty hours later, the cultures were harvested and assayed for phenylalanine hydroxylase.

| Hormone added | Final concentration of hormone | Phenylalanine hydroxylase units/lg cells |
|---------------|--------------------------------|-----------------------------------------|
| None          | 0                              | 30                                      |
| Glucagon      | 1.0 mg/ml                      | 15                                      |
|               | 1.0 μg/ml                      | 45                                      |
|               | 10.0 μg/ml                     | 58 ± 1                                  |
| Insulin*      | 10−7 M                         | 76 ± 11                                 |
|               | 10−6 M                         | 67 ± 10                                 |
|               | 10−5 M                         | 82 ± 8                                  |
| Charcoal-extracted serum* | 20% (v/v) | 145                                      |

*Where replicate cultures were assayed, the mean value ± the deviation from the mean are given.

*Insulin content of serum determined by radioimmunoassay was 14.3 microunits/ml; the final concentration of insulin in the cultures here was therefore only 2.86 microunits/ml.

- **Table II**
  - Phenylalanine hydroxylase stimulatory activities of mammalian sera after extraction with charcoal to remove endogenous glucocorticoids

- **Table III**
  - Effects of glucagon, insulin, and charcoal-extracted serum on phenylalanine hydroxylase content of basal cultures

- **Examination of Known Hormones for Their Ability to Mimic Phenylalanine Hydroxylase Stimulatory Effect of Charcoal-extracted Serum**

- **Table IV**
  - Dependence on de NOLJO Protein Synthesis of Stimulation of Phenylalanine Hydroxylase

- While the formal possibility still cannot be excluded that a subtle combination of two or more of these hormones might be acting synergistically at low levels to produce the observed effects of serum, to approach this question through an experimental protocol such as the one used above would be prohibitively complex without any prior information as to the endogenous concentrations of these hormones in serum by means of appropriate radioimmunoassays.
Comparison of phenylalanine hydroxylase stimulatory activities of charcoal-extracted serum and insulin, either alone or in combination, and effect of anti-insulin serum on stimulation by each of these agents

Experiment 1, after a 3-day precultivation in serum-free medium, replicate postconfluent cultures were given S-77 medium supplemented with either 20% (v/v) charcoal-extracted serum alone, $1.0 \times 10^{-8}$ M (146 milliunits/ml) insulin alone, or both of these agents in combination. Experiment 2, after a 4-day precultivation in serum-free medium, replicate postconfluent cultures were given S-77 medium supplemented with either 20% (v/v) charcoal-extracted serum or $1.0 \times 10^{-18}$ M (14.6 microunits/ml) insulin, and anti-insulin serum was added to the designated cultures at the final concentrations indicated in the table. Forty-four (Experiment 1) or 45 (Experiment 2) hours later, all cultures were harvested and assayed for phenylalanine hydroxylase.

The data represent the mean values and deviations from the mean obtained with duplicate sets of cultures under each experimental condition.

| Agent added          | Final concentration | Phenylalanine hydroxylase content |
|----------------------|---------------------|-----------------------------------|
|                      | Total               | Net increase                      |
|                      | units/g cells       |                                   |
| **Experiment 1**     |                     |                                   |
| None                 | 17                  |                                   |
| Insulin              | $1.0 \times 10^{-8}$ M | 61 ± 7                          | 44                       |
| Charcoal-extracted   | 20% (v/v)           | 130 ± 14                         | 113                      |
| Serum                | Insulin + charcoal-extracted serum | $1.0 \times 10^{-8}$ M | 172 ± 1                  | 105*                   |
|                      | 20% (v/v)           |                                   |                          |
| **Experiment 2**     |                     |                                   |
| None                 | 15                  |                                   |
| Insulin              | $1.0 \times 10^{-18}$ M | 39 ± 4                          |                           |
| Insulin + anti-insulin serum | $31.3$ (anti) - microunits/ml | Trace                          |                           |
| Charcoal-extracted   | 20% (v/v)           | 103 ± 2                          |                           |
| Serum                | Charcoal-extracted serum + anti-insulin serum | 50.0 (anti) - microunits/ml | 95 ± 5                    |

*If the effects of charcoal-extracted serum and insulin were numerically additive, the net increase expected would be 155 units/g.

**Cellular Phenylalanine Hydroxylase Content by Serum**—In order to examine whether or not the *de novo* synthesis of protein was required for the stimulation of cellular phenylalanine hydroxylase levels by serum, basal cell cultures were exposed to serum-free or serum-supplemented medium with or without $1 \mu$M cycloheximide, a potent inhibitor of general protein synthesis in eukaryotic cells (21). A preliminary experiment had verified that $1 \mu$M cycloheximide did not affect the incorporation of radioactive amino acid into intracellular trichloroacetic acid-soluble pools and therefore did not restrict amino acid transport into the cells.* Although this concentration of the antimetabolite does not produce a complete inhibition of protein synthesis in the H4 cells (7), $1 \mu$M was chosen because higher concentrations had been found earlier to be toxic to the cells upon overnight exposure (7). In the experiment summarized in Fig. 5, this level of the drug, while not effecting a complete inhibition of general protein synthesis in the cells, prevented the 6-fold stimulation of cellular phenylalanine hydroxylase levels by native horse serum. The small decrease in the enzyme content of the basal cultures treated with cycloheximide is of questionable significance in view of the very low enzyme levels seen with serum-free medium.

Although from the data of Fig. 5 it would appear that a 22-hour exposure to serum produced a modest increase (about 40%) in the rate of protein synthesis of basal cultures, in subsequent experiments equivalent rates of incorporation of radioactive amino acids into trichloroacetic acid-insoluble cellular material were obtained with and without serum when the labeled precursor was added to the cultures along with a complete change of medium (Table V). The simplest interpretation of these results is that cultures incubated in serum-containing medium for 22 to 27 hours consume more amino acid from the medium than do those cultivated in serum-free medium because the former cultures resume growth and therefore attain a higher population density than that of the serum-free cultures (cf. the total amounts of trichloroacetic acid-insoluble protein per culture shown in Table V), and also,
Replicate postconfluent cultures were preincubated in serum-free medium for 4 days. Then each culture received either 20 ml of S-77 medium or 16 ml of S-77 medium plus 4 ml of charcoal-extracted horse serum. After a 27-hour (Experiment 1) or a 22-hour (Experiment 2) exposure to the enzyme, the used media were removed from all flasks. One-half of the cultures (the A groups), received 5 ml each of their own used media along with 100 µl of phosphate-buffered saline containing 1.0 µCi of either \([U-^{14}C]\) phenylalanine (initial specific activity, 458 Ci/mol) (Experiment 1) or \([U-^{14}C]\) leucine (initial specific activity, 511 Ci/mol) (Experiment 2); the other half of the cultures (the B groups), received 5 ml each of fresh experimental medium supplemented with an equal amount of saline and radioactive amino acid. Duplicate (Experiment 1) or triplicate (Experiment 2) cultures were used for each experimental condition, and a single culture previously exposed to serum-free medium served as a killed-cell control for each group. After incubation for an additional 60 min at 37°C, the cultures were harvested for determination of incorporation of radioactivity into trichloroacetic acid-soluble and -insoluble cellular material according to Method B. The values given are the means from replicate cultures ± the deviations from the mean.

| Group | Serum | Total radioactivity | Specific activity of protein |
|-------|-------|---------------------|-----------------------------|
|       |       | Trichloroacetic acid | Total protein |
|       |       | -soluble            | -insoluble                  | |
|       |       | dpm x 10^4/culture | mg/culture | dpm x 10^4/mg |
| Experiment 1 | | |
| A | - | 1.03 ± 0.022 | 15.5 ± 0.40 | 5.57 ± 0.07 | 2.78 |
| B | + | 1.00 ± 0.044 | 33.4 ± 0.44 | 6.04 ± 0.06 | 4.83 |
|   | - | 1.03 ± 0.052 | 23.2 ± 0.40 | 5.63 ± 0.13 | 4.15 |
|   | + | 1.11 ± 0.041 | 27.7 ± 0.32 | 6.68 ± 0.06 | 4.14 |
| Experiment 2 | | |
| A | - | 1.12 ± 0.066 | 24.5 ± 0.20 | 4.30 ± 0.00 | 5.69 |
| B | + | 1.46 ± 0.079 | 55.8 ± 0.95 | 5.26 ± 0.00 | 6.89 |
|   | - | 1.33 ± 0.040 | 14.7 ± 0.77 | 4.40 ± 0.00 | 3.33 |
|   | + | 1.48 ± 0.100 | 16.7 ± 0.23 | 5.55 ± 0.12 | 3.14 |

* Value for serum-stimulated cultures divided by value for basal cultures.

Discussion

The effects of serum on cultured animal cells are manifold and complex; indeed, only a few established cell lines are capable of replication, or even survival, in culture without serum or some macromolecular component derived from serum (19, 22–31). Thus, within the context of the diverse biological activities of serum, a major concern in attempting to evaluate the significance of an effect of serum on any given cellular function is the extent to which that effect is specific for the function in question. The experimental evidence presented here is consistent with the following conclusions.

Cultivation of H4-II-E-C3 cells in serum-free medium results in an exponential decay of their phenylalanine hydroxylase content to values that probably reflect the constitutive level of expression of the phenylalanine hydroxylase gene in these cells. Re-exposure of the resulting basal cultures to serum evokes a stimulation of their enzyme content that is both time- and dosage-dependent and requires protein synthesis. The stimulatory factor in serum is not dialyzable and is stable at 65°C for 15 min. Evidence that serum contains a phenylalanine hydroxylase stimulatory component(s) in addition to the endogenous glucocorticoids was garnered from three observations: (a) glucocorticoid antagonists ("anti inducer" steroids) inhibited less than one-half of the biological activity of serum in the cell culture system; (b) serum that had been exhaustively extracted with charcoal still retained about one-half of the phenylalanine hydroxylase stimulatory activity of native serum and this activity was not antagonized by the anti-inducer fluoxymesterone; and (c) the effects of maximally stimulatory concentrations of charcoal-extracted serum and hydrocortisone were additive. From the last of these findings, it follows that both glucocorticoids and the active component(s) in serum are required for the maximum expression of cellular phenylalanine hydroxylase levels. A survey of partially purified preparations of a number of known hormones failed to reveal any one capable of elevating the phenylalanine hydroxylase levels of basal cultures in a manner comparable to that of charcoal-extracted serum. Finally, whereas the phenylalanine

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**Table V**

**Effect of exposure of basal cultures to serum on their incorporation of radioactive amino acids into trichloroacetic acid-soluble and -insoluble material**

| Group | Serum | Total radioactivity | Specific activity of protein |
|-------|-------|---------------------|-----------------------------|
|       |       | Trichloroacetic acid | Total protein |
|       |       | -soluble            | -insoluble                  | |
|       |       | dpm x 10^4/culture | mg/culture | dpm x 10^4/mg |
| Experiment 1 | | |
| A | - | 1.03 ± 0.022 | 15.5 ± 0.40 | 5.57 ± 0.07 | 2.78 |
| B | + | 1.00 ± 0.044 | 33.4 ± 0.44 | 6.04 ± 0.06 | 4.83 |
|   | - | 1.03 ± 0.052 | 23.2 ± 0.40 | 5.63 ± 0.13 | 4.15 |
|   | + | 1.11 ± 0.041 | 27.7 ± 0.32 | 6.68 ± 0.06 | 4.14 |
| Experiment 2 | | |
| A | - | 1.12 ± 0.066 | 24.5 ± 0.20 | 4.30 ± 0.00 | 5.69 |
| B | + | 1.46 ± 0.079 | 55.8 ± 0.95 | 5.26 ± 0.00 | 6.89 |
|   | - | 1.33 ± 0.040 | 14.7 ± 0.77 | 4.40 ± 0.00 | 3.33 |
|   | + | 1.48 ± 0.100 | 16.7 ± 0.23 | 5.55 ± 0.12 | 3.14 |

* Value for serum-stimulated cultures divided by value for basal cultures.
hydroxylase stimulatory activities of various mammalian sera in the native state were variable, the activities of the same sera after charcoal extraction were indistinguishable from one another.

The data presented in Table V and Fig. 5 suggest that the effect of serum on cellular phenylalanine hydroxylase content, while requiring concomitant protein synthesis, cannot be attributed to a nonspecific increase in protein anabolism. In this connection, it may be noted that, in marked contrast to the pronounced pleiotropic effects of serum on 3T3 fibroblasts (32), a serum “step-down” from 10 to 0.5% for up to 72 hours caused little decrease, if any, in the rate of cellular protein synthesis in BHK 21/13 fibroblasts, as measured both by the incorporation of labeled valine into cultures and by the protein synthetic activity of cell extracts in a cell-free system in vitro (33). On the other hand, in hepatoma tissue culture cells preincubated in serum-free medium plus dexamethasone for 16 hours, after exposure to 5% bovine serum for 1 hour, the rate of incorporation of labeled amino acid into total cellular protein was increased by 43% (34) and the rate of total protein degradation decreased by 18% (35).

A comparison of the effects of serum on tyrosine aminotransferase and phenylalanine hydroxylase in cultured rat hepatoma cells warrants some discussion. First, whereas in hepatoma tissue culture (34) cells the stimulatory effects of serum and dexamethasone on tyrosine aminotransferase are additive, the stimulation by serum requires the continuous presence of dexamethasone. In contrast, an increase in the phenylalanine hydroxylase levels of H4 cells can be effected by serum in the absence of glucocorticoids (Fig. 4). Conversely, through the use of a strain of RLC cells (RLC-GAI) adapted to growth in serum-free medium (19), Gerschenson et al. have shown that the paradoxical elevation by actinomycin D of tyrosine aminotransferase activity in dexamethasone-preincubated cells (i.e. “superinduction”) requires serum (36). It would be of interest to ascertain whether or not superinduction by actinomycin D, if also seen with the phenylalanine hydroxylase system of H4 cells, would likewise be serum-dependent. Finally, the tyrosine aminotransferase stimulatory activity of bovine serum, like the phenylalanine hydroxylase stimulatory activity of horse serum, was moderately heat-stable and nondialyzable (34).

The following lines of evidence would argue that serum and hydrocortisone elevate the phenylalanine hydroxylase levels of H4 cells by increasing the amount of phenylalanine hydroxylase protein and not by activating the enzyme, either directly or indirectly: (a) the effects of these agents are not detectable before 8 to 10 hours of exposure; (b) both effects require continuous protein synthesis; (c) neither agent has any effect on the phenylalanine hydroxylase activity of cell extracts when added to the enzyme-assay mixture in vitro; (d) a mixture of extracts from basal and from serum plus hydrocortisone-stimulated cells contained the phenylalanine hydroxylase levels expected on the basis of numerical additivity; and (e) through the use of sheep-phenylalanine hydroxylase serum, generously provided by Dr. Seymour Kaufman (37), extracts from cells exposed either to charcoal-extracted horse serum alone or to hydrocortisone alone were found to contain far greater amounts of immunoprecipitable material than did extracts from basal cells, as visualized by Ouchterlony double immunodiffusion.  

It remains to be determined whether each of these agents elevates the amount of phenylalanine hydroxylase in the cells by increasing the rate of synthesis of the enzyme, decreasing the rate of its degradation, or both. In this connection, if the rates of formation and decay of enzyme are assumed to be zero and first order, respectively, in accordance with the model first proposed by Schimke (40), the fact that the half-times of stimulation and decline of cellular phenylalanine hydroxylase content upon exposure to charcoal-extracted serum (Fig. 2) and upon withdrawal of serum (Fig. 1), respectively, are comparable (about 22 hours) would support the first of these alternatives with respect to serum.

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*1. The factor contained in the sera which stimulates the levels of phenylalanine hydroxylase in the cultured cells is clearly distinct from the rat liver protein which stimulates the activity of phenylalanine hydroxylase in vitro (38, 39).

**2. The factor contained in the sera which stimulates the levels of phenylalanine hydroxylase in the cultured cells is clearly distinct from the rat liver protein which stimulates the activity of phenylalanine hydroxylase in vitro (38, 39).
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