Regulation of Tyrosine \(\alpha\)-Ketoglutarate Transaminase in Rat Liver

VIII. INDUCTIONS BY HYDROCORTISONE AND INSULIN IN CULTURED HEPATOMA CELLS*

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SUMMARY

Hydrocortisone addition to cell cultures of the Reuber (H-35) hepatoma initiated an 8- to 10-fold increase in tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) levels, the response being maximal at 9 hours and persisting at this new level for at least 36 hours. The optimal concentration of hydrocortisone for this induction was \(5 \times 10^{-7} \text{M}\). Insulin was also found to elevate this enzyme in H-35 cells, and caused the enzyme to rise without the lag period that is apparent after hydrocortisone. With a single addition of insulin, the increase in enzyme (5- to 6-fold) ceased after 8 hours, but a high steady state could be maintained by resupplementation with insulin. When the effect of insulin is measured at 6 hours, 1 milliunit per ml is optimal and 0.02 milliunit per ml is readily detected. The isolated A and B chains of insulin are not effective and do not compete with intact insulin. Glucagon is not effective in these cells, which appear to lack adenyl cyclase activity. Immunochemical-isotopic analyses show that both hydrocortisone and insulin specifically accelerate synthesis of the enzyme, but there may also be a stimulation of general protein synthesis after insulin treatment. Analysis of the kinetics of these inductions indicates a half-life of about 3 hours for tyrosine transaminase in the cells; this was confirmed by "chase" analysis and shown to be unaffected by insulin. Transaminases from hydrocortisone-induced, insulin-induced, and noninduced cells are immunologically identical with the rat liver enzyme.

In previous papers of this series it was established that adrenal glucocorticoids and the pancreatic hormones, insulin and glucagon, are all capable of stimulating the synthesis of tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) in rat liver in vivo (1–3). In experiments with isolated, perfused livers it was shown that each of these hormones acts independently of the others (4); this conclusion has been reinforced by Wicks, in studies involving organ cultures of fetal liver (5). In a number of reports, Tomkins and his colleagues have described several facets of induction by glucocorticoids in a rat hepatoma cell line (HTC) maintained in cell culture (6–16). We here describe the results of our analyses of some parameters of induction of tyrosine transaminase by hydrocortisone and by insulin in a similar, but not identical, rat hepatoma cell culture referred to as the H-35 or Reuber hepatoma cell line. These cells were first adapted to culture and analyzed by Pilote et al. (16) and originated from a minimal deviation tumor arising from aminomethylfluorene treatment in studies by Reuber (17).

EXPERIMENTAL PROCEDURE

Hepatoma Cells—The cells were grown as monolayers in 75-cm² plastic flasks as previously described (18). During growth the basal medium of Eagle was enriched 4-fold with vitamins and amino acids and also supplemented with 20% fetal calf serum and 0% calf serum. All experiments were performed with either 4- to 5-day (log phase) or 9- to 12-day (stationary phase) cultures maintained for 24 hours in serum-free, unenriched Eagle's basal medium. The hormone effects described do not differ appreciably in log phase or stationary phase cells. H-35 cells do not grow in serum-free BME but will resume growth on readdition of serum after as long as 6 days in its absence. In suspension culture experiments, cells were scraped from monolayers with a rubber policeman, collected by low speed centrifugation, and suspended in BME in a rotary shaker oscillating at 100 rpm. All experiments were performed with either 4- to 5-day (log phase) or 9- to 12-day (stationary phase) cultures maintained for 24 hours in serum-free, unenriched Eagle's basal medium. The hormone effects described do not differ appreciably in log phase or stationary phase cells. H-35 cells do not grow in serum-free BME but will resume growth on readdition of serum after as long as 6 days in its absence.

Materials—All tissue culture materials were purchased from Grand Island Biologicals except penicillin G and streptomycin sulfate, which were obtained from Squibb Pharmaceuticals. All labeled compounds used in these experiments were purchased from New England Nuclear, hydrocortisone (Grade A) was from

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Calbiochem, insulin (24 units per mg) was from Sigma, the isolated A and B chains of insulin were from Mann, and anti-insulin serum was from Pentex.

**Enzyme Assay**—Hepatoma cells were broken either by sonic oscillation in cold 0.15 M KCl-0.001 M EDTA, pH 8.0, or by lysis in this medium with added 0.038 mM pyridoxal phosphate and 5.0 mM α-ketoglutarate, pH 7.0. For sonic oscillation, cell suspensions held in an ice bath were treated with a Biosonik probe sonicator (Bronwill Scientific) with a single 15-sec burst at the minimum probe setting. Cell lysis was achieved by alternately freezing and thawing three times in liquid nitrogen and a 37° water bath, respectively. Cells from the same flask, broken by either sonic oscillation or lysis, yielded essentially identical enzyme activity. Sonically disrupted extracts of whole cells, or supernatant fractions of cell lysates centrifuged at 105,000 × g for 1 hour, were assayed directly for tyrosine transaminase activity with the assay conditions described by Kenney (19) and the product formation test of Diamondstone (20). One unit of activity is that amount required to form 1 μg of p-hydroxyphenylpyruvate during a 10-min incubation period. Protein was estimated by the procedure of Lowry et al. (21).

**Immunochemical Analysis**—In experiments in which rates of enzyme synthesis or degradation were measured, monolayer cultures were exposed to isotopic leucine for varying intervals, as described in individual legends. Incorporation of isotope into tyrosine transaminase was measured immunologically as described previously (18).

**RESULTS AND DISCUSSION**

**Growth of Reuber H-35 Hepatoma in Tissue Culture**—Fig. 1 shows the current growth curve for cells of the Reuber H-35 hepatoma, which propagate to form multilayered confluent sheets on glass or plastic surfaces. Inoculation of 1.3 × 10⁵ cells per ml of growth medium resulted in cultures which entered stationary phase on about Day 9. The doubling time during logarithmic growth is about 24 hours, whereas this cell line had a doubling time of 3.2 days when originally placed in culture (16). Although the growth rate of the Reuber H-35 hepatoma in cell culture has thus increased appreciably since its initial culturing, these cells have retained their ability to respond to some of the hormones which increase tyrosine transaminase levels in vivo. The HTC cell line, in which tyrosine transaminase is also inducible, also has a doubling time of 24 hours (6).

**Tyrosine Transaminase Induction by Hydrocortisone**—Addition of hydrocortisone (10⁻⁸ M) to suspension cultures of H-35 hepatoma cells elicited an 8-fold increase in tyrosine transaminase levels, the response being maximal before 12 hours and persisting for as long as 24 hours (Fig. 2A). The absolute enzyme level reached at maximal induction varied to some extent from one experiment to another, apparently reflecting minor variations in prior treatment of the cells. If the steroid-containing medium was replaced with fresh steroid-free medium following maximal induction (Fig. 2B), there was a 1- to 2-hour period of little change, after which the enzyme slowly declined toward basal levels. Similar time courses of glucocorticoid induction and decay of enzyme activity following steroid removal have also

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Growth curve for H-35 hepatoma cells in monolayer culture. Flasks were inoculated with 1.3 × 10⁵ cells per ml and fed every other day by replacing half of the growth medium (BME enriched 4-fold with amino acids and vitamins and supplemented with 20% fetal calf and 5% calf serum). Each day the cells in three flasks were detached by trypsinization and counted in a Coulter cell counter. Each point represents the mean of cell counts from three individual flasks.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Kinetics of induction and decay of tyrosine transaminase following addition or removal of hydrocortisone in suspension cultures of H-35 hepatoma cells. A, induction: cells were scraped from monolayer and incubated at 37° in 100-ml suspension cultures with (●—●) and without (O—O) 10⁻⁸ M hydrocortisone. B, decay: 100-ml suspension cultures were previously induced for 24 hours with 10⁻⁸ M hydrocortisone. At zero time the medium was replaced with medium containing 10⁻⁸ M hydrocortisone (●—●) or without added hydrocortisone (O—O). In both A and B, 10-ml samples of cells were taken at the times shown for assay of enzyme activity.
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FIG. 3. Effect of varying concentration of hydrocortisone on tyrosine transaminase levels in H-35 cells. Induction time was 6 hours.

been observed in cultures of HTC cells (6, 11, 14). It is of interest that these two cell lines, maintained in culture, appear to be unable to metabolize glucocorticoids as effectively as the normal intact liver, since the hormone must be administered repeatedly in order to maintain this induction in vivo (22).

It will be noted from Fig. 1A that the basal enzyme level tends to increase slightly when the cells are exposed to fresh medium at the start of the experiment. This has been traced to small shifts in the pH of the medium, and can be prevented if the medium is gassed with CO₂ for a few minutes after its addition to the monolayers (cf. Fig. 9). When this effect on the basal enzyme level is considered, it is apparent that hydrocortisone has virtually no effect during the 1st hour; thus the pronounced lag apparent in similar experiments in vivo (2) is also found in the cell culture system.

Analyses of the quantity of steroid required to increase the transaminase level showed that a concentration of 5 × 10⁻⁷ M was optimal, while 10⁻⁸ M yield a detectable response (Fig. 3). Circulating levels of glucocorticoids in humans have been estimated to be about 3 × 10⁻⁷ M (23). Thus the response observed here is clearly attuned to "physiological" hormone levels.

Induction by Insulin—The time course of change in activity of tyrosine transaminase after addition of insulin (1 milliunit per ml) to H-35 cells is shown in Fig. 4. A significant increase was apparent within 0.5 hour, comparable to the rapid response to insulin in vivo (3) but in sharp contrast to the pronounced lag following hydrocortisone treatment in vivo (2) or in these cultures (Fig. 2A). At this level of insulin the transaminase continued to increase for a period of 7 hours, after which the enzyme level gradually declined. This decline is apparently due to lability or metabolism of the hormone since the peak level could be maintained by resupplementation of the medium with insulin (cf. Fig. 9).

The amount of insulin required to effect induction is very small—1 milliunit per ml (approximately 6 × 10⁻⁸ M) yielding a maximal response, and 0.04 milliunit per ml (approximately 10⁻⁹ M) being clearly detectable—when induction was measured at 6 hours (Fig. 5).

The isolated A and B chains of insulin had no effect on the transaminase level, whether tested alone or in combination. Similarly, these polypeptides did not inhibit the effect of intact insulin (Table I).
TABLE I
Effect of insulin A chain, insulin B chain, and insulin on tyrosine transaminase levels in H-35 cells

The concentrations of insulin, insulin A chain, and insulin B chain were 1 milliunit (0.042 μg) per ml, 1 μg per ml, and 1 μg per ml, respectively. Induction time was 5 hours. Data are the mean ± standard error; number of observations is in parentheses.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Tyrosine transaminase units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>32 ± 1 (0)</td>
</tr>
<tr>
<td>Insulin</td>
<td>130 ± 4 (5)</td>
</tr>
<tr>
<td>Insulin A chain</td>
<td>33 ± 1 (5)</td>
</tr>
<tr>
<td>Insulin B chain</td>
<td>32 ± 1 (5)</td>
</tr>
<tr>
<td>Insulin A chain and insulin B chain</td>
<td>34 ± 1 (3)</td>
</tr>
<tr>
<td>Insulin and insulin A chain</td>
<td>132 ± 6 (4)</td>
</tr>
<tr>
<td>Insulin and insulin B chain</td>
<td>134 ± 4 (3)</td>
</tr>
<tr>
<td>Insulin, insulin A chain, and insulin B chain</td>
<td>141 ± 6 (3)</td>
</tr>
</tbody>
</table>

TABLE II
Effect of anti-insulin serum on glucagon or insulin effects of H-35 cells

Where indicated, hormones were previously treated for 30 min at 37° with 2 μl per μg (glucagon) or 2 μl per milliunit (insulin) of either a control rabbit serum or a rabbit antisemur prepared against insulin (titer: 1 unit per ml). These preparations were then diluted to the concentrations indicated before being added to the cultures. Two glucagon preparations from commercial sources (A and B) were used. Data are the mean ± standard error for three or four observations; the induction time was 5 hours.

<table>
<thead>
<tr>
<th>Hormone addition</th>
<th>Serum treatment</th>
<th>Tyrosine transaminase units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Glucagon A (10 μg/ml)</td>
<td>None</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Glucagon A (10 μg/ml)</td>
<td>Control</td>
<td>108.6 ± 4</td>
</tr>
<tr>
<td>Glucagon B (50 μg/ml)</td>
<td>Control</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Glucagon B (50 μg/ml)</td>
<td>Anti-insulin</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Insulin</td>
<td>Control</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>1 milliunit/ml</td>
<td>Anti-insulin</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>0.02 milliunit/ml</td>
<td>Control</td>
<td>76 ± 4</td>
</tr>
<tr>
<td></td>
<td>Anti-insulin</td>
<td>27 ± 1</td>
</tr>
</tbody>
</table>

FIG. 6. Immunological titration of transaminase preparations. Crude enzyme preparations (105,000 × g supernatant fractions) from hydrocortisone-treated (○), insulin-treated (△), or control (●) H-35 cells were titrated against antitransaminase serum sufficient to precipitate 166 units of transaminase from a fresh rat liver-soluble fraction. The mixtures were incubated at 4° overnight and centrifuged to remove the enzyme-antibody complex before assay for transaminase remaining soluble. The crude enzyme preparations had specific activities (units per mg of protein) of 21, 125, 224 for control, insulin-treated, and hydrocortisone-treated cells, respectively.

Apparent Effect of Glucagon—In preliminary reports of this work (24, 25) some effects of glucagon on the tyrosine transaminase levels of H-35 cells were described. Some of the glucagon preparations used were specifically treated to remove contaminating insulin. Nevertheless, the very low concentrations of insulin required (Fig. 5) demanded a re-examination of the apparent effect of glucagon; this was done with anti-insulin serum (Table II). The results of these experiments make it clear that the effects of the glucagon preparations used were, in fact, due to contaminating insulin. Adenyl cyclase assays of H-35 cell homogenates and the fraction sedimenting at 600 × g yielded borderline results, suggesting the absence of this enzyme, as has been reported for the similar HTC line (26).

Supplementation with glucagon did not increase the negligible cyclase activity of the H-35 cells. Nevertheless, the dibutylly analogue of cyclic AMP is effective in increasing the transaminase level of these cells; studies of the action of this nucleotide are currently in progress.

Immunological Analyses—Previous studies have shown that the tyrosine transaminase induced in vivo is immunologically identical with the basal enzyme, whether induced by hydrocortisone or by insulin (1, 3). Similar results were obtained in analysis of this point in the hormone-treated H-35 cells (Fig. 6). Here crude enzyme preparations from untreated, insulin-treated, and hydrocortisone-treated cells were titrated against a constant amount of antitransaminase serum, prepared against enzyme purified from hydrocortisone-treated rate. All of the preparations yielded the same equivalence point, indicating both that the antibodies are immunologically identical (within the limitations of this technique) and that the concentration of enzyme changes to an extent equivalent to the activity changes measured in other experiments. The recent suggestion that there may exist separate enzymes which respond differentially to various hormonal inducers (27) is not substantiated by these results. By the immunological criterion used here, treatment of H-35 cells with either the steroid hydrocortisone or the polypeptide insulin increases the concentration of the same tyrosine transaminase present in untreated cells.

The tyrosine transaminase of H-35 cells undergoes a rapid turnover (18), as does in the liver in vivo (28); we have demonstrated that the level of this enzyme can be elevated by blocking the degradative process, as well as by stimulation of synthesis (18). To determine which of these processes is affected by hydrocortisone and by insulin, isotopic labeling of the enzyme...
TABLE III

Effect of hydrocortisone on synthesis of tyrosine transaminase in H-35 cultures

Medium with or without hydrocortisone (10^{-4} M) was placed on monolayer cultures 30 hours (Experiment 1) or 3 hours (Experiment 2) before the cells were collected for analysis. 3H-Leucine (5 pCi per ml, Experiment 1, 10 pCi per ml, Experiment 2) was added 15 min before cells were collected. Cells from seven monolayer cultures were pooled for each analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hrs)</th>
<th>Transaminase activity</th>
<th>Radioactivity in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>units/mg protein</td>
<td>cpm</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>33</td>
<td>450</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>30</td>
<td>347</td>
<td>3,505</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>39</td>
<td>113</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>3</td>
<td>93</td>
<td>616</td>
</tr>
</tbody>
</table>

Followed by isolation with antitransaminase serum was used. In short exposure or pulse experiments, incorporation of isotope into the enzyme is a measure of its rate of synthesis. The experiments reported in Table III, with this criterion, show that increased transaminase levels attained after hydrocortisone treatment are the result of increased synthesis of the enzyme. In the first experiment, measurements were made well after the induced steady state was reached; both the amount of enzyme and its rate of synthesis were increased about 10-fold. In this experiment there appeared to be an effect of hydrocortisone on labeling of the total soluble proteins. This is not generally observed in making these measurements, and may reflect a cytotoxic effect of long term hydrocortisone treatment in these cultures, resulting in depletion of amino acid pools. In the second experiment, in which measurements were made shortly after induced synthesis began, this effect was not seen. Transaminase synthesis was increased about 6-fold, but the enzyme level was only doubled at this early point in the response. These results confirm similar analyses of the hydrocortisone effect of tyrosine transaminase in vivo (2) as well as in the ITC cell line (11).

Fig. 7. Effect of insulin on rate of transaminase synthesis. Insulin (1 milliunit per ml) was added at zero time to seven monolayer cultures for each time point (O—O); a comparable number of cultures to which no insulin was added served as controls ( — — ). 3H-Leucine (100 pCi per ml, 250 mCi per mmole) was added 15 min before collection of cells for analysis. A, transaminase activity; B, specific transaminase radioactivity (counts per min in transaminase per cpm per mg of total soluble protein); C, radioactivity in total soluble protein.

Fig. 8. Rate of transaminase degradation in control and insulin-treated H-35 cells. 3H-Leucine (0.5 pCi per ml, 250 mCi per mmole) was added 6.5 hours before measurements began to seven monolayer cultures for each point; insulin was also added to some of these (— — ) and omitted from controls ( — — ). At zero time the isotope-containing medium was decanted and the monolayers were washed three times with 5 ml of medium. During the time course shown, the cells were incubated at 37° in unlabeled medium with (— — ) and without ( — — ) insulin. Cells were collected and pooled for analysis at the time indicated. Transaminase activity increased appreciably during this interval in insulin-treated cells (113 to 142 units per mg of protein) and slightly in control cells (17 to 25 units per mg of protein). The lines drawn were calculated by the method of least squares.

Fig. 9. Effect of hydrocortisone plus insulin on tyrosine transaminase levels in H-35 cells. Hormone additions were: none, O—O; insulin (1 milliunit per ml) at zero time and again at 7 hours, V—V; hydrocortisone (10^{-4} M) at zero time, A—A; hydrocortisone plus insulin as above, — — .
Comparable experiments carried out with insulin as inducer revealed a more complicated cell response. There was clearly an effect on the rate of labeling of the total soluble proteins, becoming more extensive as the time of insulin treatment increased (Fig. 7C). From labeling data alone it cannot be distinguished whether this reflects insulin effects on permeability and consequent changes in radioactivity of intracellular amino acid pools or an actual stimulation of general protein synthesis.

Measurements of the differential effect of insulin on transaminase synthesis (Fig. 7B), compared to the increase in amount of transaminase (Fig. 7A), support the view that stimulation of labeling of the soluble proteins does reflect a nonspecific increase in protein synthesis. Thus, except at early time points after insulin addition, the increase in specific transaminase labeling is not sufficient to account for the extent of increase in the amount of enzyme. These results can be understood as indicating that the increase in transaminase level reflects both a specific acceleration of enzyme synthesis and accumulation of enzyme due to a general effect of insulin on protein synthesis. It may be that synthesis of tyrosine transaminase is, for reasons not understood, particularly sensitive to a general effect of insulin on some component of the protein-synthesizing mechanism. Wool, Martin, and Low (29) have described such an effect of insulin on muscle ribosomes.

Since our results might also be interpreted as suggesting an effect of insulin on enzyme turnover, the possibility that insulin alters the rate of degradation of the enzyme was examined. Cultures were previously treated with insulin and 14C-Leucine (18). In control cells treated similarly with isotopic leucine, and unlabeled leucine. Under these "chase" conditions, loss of radioactivity from the enzyme is a measure of its rate of degradation. The half-life of the enzyme determined in this fashion was about 3 hours (Fig. S), comparable to that determined previously in hydrocortisone-treated H-35 and HTC cells (18). In control cells treated similarly with isotopic leucine but not treated with inducing hormones, the rate of transaminase degradation was similar to that determined in the presence of insulin (Fig. S); thus, there is no indication of a marked effect of insulin (or of hydrocortisone) on enzyme turnover.

Additivity of Hydrocortisone and Insulin—The effects of each of these hormones singly, and in combination, on tyrosine transaminase levels are shown in Fig. 9. In these experiments the medium was resupplemented with insulin at 7 hours in order to counteract the apparent lability of the hormone under these conditions (cf. Fig. 4). Under these conditions it is apparent that the effects of the two hormones together are synergistic, implying (as do the different kinetics of response) that they act by different mechanisms. This is discussed in more detail in the next paper in this series (30).

It will be noted that the induced steady state is reached approximately 9 hours after the induction process begins, regardless of the nature of the inducing agent or even the combination of agents. This is in accord with the theoretical treatment elaborated by Berlin and Schimke (31), which indicates that the time required to reach a new steady state (after a stimulation of synthesis) is dependent upon the rate at which the protein involved undergoes degradation. These results, then, lend further emphasis to the conclusion that the rate of degradation of tyrosine transaminase is unchanged by these hormones. Further, they permit an independent estimate of that rate, since the time required to reach 50% of the total change is equivalent to the half-life of the enzyme (31). By this criterion, also, the half-life of the enzyme is about 3 hours.

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