*"SUPERINDUCTION" OF TYROSINE TRANSAMINASE IN HEPATOMA CELL CULTURES: DIFFERENTIAL INHIBITION OF SYNTHESIS AND TURNOVER BY ACTINOMYCIN D**

By Jerry R. Reel† and Francis T. Kenney

NCI-AEC CARCINOGENESIS PROGRAM, BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Communicated by William A. Arnold, July 12, 1968

Continuous breakdown and replenishment of the macromolecular constituents of animal cells was recognized some time ago in the pioneering experiments of Schoenheimer and his colleagues,[1] but the significance of degradative processes in regulation of metabolism has become apparent only recently. Schimke, Sweeney, and Berlin have demonstrated[2] that the physiological level of the enzyme tryptophan pyrrolase can be elevated either by stimulation of its synthesis or by inhibition of its degradation, the former process being initiated by glucocorticoid hormones and the latter by the substrate of the enzyme, tryptophan. In a recent report[3] from our laboratory, it was shown that degradation of the tyrosine transaminase of liver was blocked when protein synthesis was inhibited by agents such as cycloheximide or puromycin; under these conditions, the transaminase level was stabilized by concomitant inhibition of both synthesis and degradation of the enzyme. At that time it was suggested[3] that certain "paradoxical" effects of inhibitors of RNA or protein synthesis on enzyme levels might result from differential inhibition of the cellular processes involved in forming an enzyme and of those required for its removal. In the present study, we have analyzed the roles of synthesis and degradation in the elevation of tyrosine transaminase, which follows the addition of actinomycin D to hormonally induced cell cultures. We find that transaminase synthesis, initially high due to induction by hydrocortisone, is progressively inhibited by the antibiotic. That the transaminase level rises during this interval, despite inhibition of its synthesis, reflects a marked inhibition of degradation of the enzyme.

Materials and Methods.—Hepatoma cultures of the Reuber H-35[4] and hepatoma tissue culture (HTC)[5] cell lines were grown in monolayer in 250-ml Falcon plastic flasks containing 10 ml of Eagle's basal medium (BME) enriched fourfold with amino acids and vitamins and supplemented with 20% fetal calf serum and 5% calf serum. Penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml) were added for routine culturing. Occasionally, antibiotics have been omitted for several passages and the medium cultured for bacterial contamination. Checks for pleuropneumonia-like (PPLO) contamination also have been carried out using the method of Barile, Yaguchi, and Eveland[6] but with horse serum in place of whole blood in the plating agar. No contamination by bacteria or mycoplasmas has been detected. All tissue culture materials were purchased from Grand Island Biological, Co. except penicillin G and streptomycin sulfate, which were obtained from Squibb.

During logarithmic growth, both cell lines had a doubling time of approximately 24 hr. Inoculation of cells at a concentration of 1.3×10^5 cells/ml resulted in cultures which enter stationary phase on about day 9. All experiments were performed using 9- to 12-day (stationary phase) monolayer cultures in serum-free 1X BME (i.e., unenriched). Reuber H-35 and HTC 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.

Hydrocortisone (Calbiochem) was dissolved in a minimal volume of ethanol and diluted

100-fold with Hanks' balanced salt solution (HBSS) to give a final stock concentration of 10^{-4} *M*. A medium concentration of 10^{-6} *M* hydrocortisone was employed to preinduce tyrosine transaminase, since this concentration was found to be the optimal inducing level in these cultures[7] as well as in organ cultures of fetal liver.[8] Actinomycin D, a generous gift from Merck and Co., was dissolved in a minimal volume of acetone and brought to 0.5 mg/ml with HBSS. Aliquots of a freshly prepared actinomycin D solution were added to the culture medium, as required, in each experiment.

Enzyme assays: Hepatoma cells were lysed in 0.15 *M* KCl–0.001 *M* ethylenediaminetetraacetate (EDTA), pH 8.0, containing 0.038 mM pyridoxal phosphate and 5.0 mM α-ketoglutarate, pH 7.6. Cell lysis was achieved by alternately freeze-thawing three times in liquid nitrogen and 37°C, respectively. The 105,000 *g* supernatant fraction of the lysates was assayed directly for tyrosine transaminase activity by a combination of the methods of Kenney[9] and Diamondstone.[10] The unit of activity is defined as that amount required to form 1 μg of p-hydroxyphenyl pyruvate during a 10-min incubation period. Protein was estimated by the procedure of Lowry *et al.*[11]

Immunochemical analysis: The rates of enzyme synthesis and degradation were measured immunochemically by a combination of the methods employed by Kenney,[12] Segal and Kim,[13] and Schimke, Sweeney, and Berlin.[2] In experiments measuring rates of synthesis, monolayer cultures were exposed to 5.0 μc or 7.5 μc of 4,5-H^3-leucine (specific radioactivity 5045 mc/mole) per ml of BME during the final 15 min of the treatment period. Since this labeling time is short relative to the half life of the enzyme, the contribution of turnover is negligible, and the extent of isotope incorporation into the enzyme is a measure of its rate of synthesis. The rate of degradation was measured by following the "chase" of H^3-leucine or C^{14}-leucine from the prelabeled enzyme. Monolayer cultures were exposed either to 5.0 μc of H^3-leucine or to 0.5 μc of U-C^{14}-leucine (specific radioactivity 273 mc/mmole) per ml of BME during the final hour of the 24-hr preinduction period. At the end of the 1-hr labeling period, the H^3- or C^{14}-leucine-containing medium was decanted, the monolayers were washed with HBSS, and then fresh BME medium containing unlabeled leucine and other appropriate treatments was added ("chase" medium). At the end of the pulse label, or "chase" periods, groups of monolayer cultures were scraped, pooled, and collected by centrifugation. The pelleted cells were washed with cold 0.15 *M* NaCl, lysed by freeze-thawing, and the lysates centrifuged at 105,000 *g* for 1 hr. Aliquots of the 105,000 *g* supernatant fraction were assayed for enzyme activity, soluble protein, and radioactivity in the soluble protein. The remainder of the 105,000 *g* supernatant fraction was rapidly heated to 60°C in the presence of 0.038 mM pyridoxal phosphate and 5.0 mM α-ketoglutarate, coenzyme and substrate, respectively, which stabilize tyrosine transaminase to heat. After being heated, supernatant fractions were rapidly cooled to 0°C and centrifuged at 20,000 *g* for 30 min. The supernatant enzyme was precipitated with antiserum prepared against a highly purified transaminase preparation.[12] The protein content of the 20,000 *g* supernatant fraction was essentially constant, but the enzyme content varied according to the treatment given (100–2000 units). Unlabeled carrier enzyme from induced rat livers (spec. act. 12,500 units/mg protein, purified through the $(NH_4)_2SO_4$ [II] step in the scheme described previously[12]) was added to each to bring the enzyme level to a constant value (25,000 units). A slight excess of antibody was added and the mixture incubated at 3°C overnight. Precipitates were collected, washed, and counted as described previously.[12] After removal of the precipitate, the supernatant fractions contained less than 2% of the original enzyme activity. To correct for nonspecific precipitation of radioactivity,[14] a second incubation was carried out in which the enzyme level of the same supernatant fraction was again brought to 25,000 units by addition of unlabeled carrier enzyme. After precipitation with antibody as before, the precipitate was collected and its radioactivity was determined. Radioactivity of the second precipitate was subtracted from the value obtained in the first precipitation, the difference being the actual extent of transaminase labeling. Labeling of the total soluble proteins was assessed by counting an aliquot that was pipetted onto filter paper disks and washed as described before.[15]

Results.—Effect of actinomycin D on the tyrosine transaminase level and its rate of synthesis and degradation: Figure 1 shows the time course of actinomycin D effects on the enzyme level and the rate of enzyme synthesis in preinduced monolayer cultures of the Reuber H-35 hepatoma. In cultures receiving hydrocortisone plus actinomycin D, the enzyme level began to rise after one hour, reached a peak at three hours, and thereafter fell slightly. During this interval, actinomycin D treatment resulted in an exponentially *decreasing* rate of enzyme synthesis, presumably reflecting the functional decay of the messenger RNA (mRNA) for tyrosine transminase ($t_{1/2}$ about 3 hr).

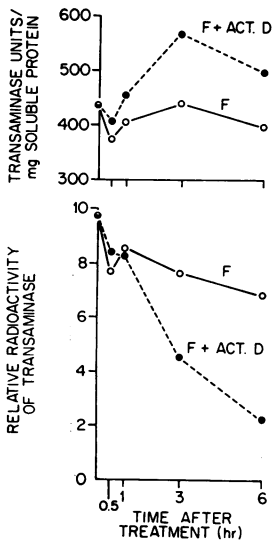

FIG. 1.—Effect of actinomycin D on the time course of transaminase synthesis in preinduced Reuber H-35 cultures in stationary phase. Medium containing hydrocortisone (10^{-6} *M*) was placed on the monolayers 24 hr before the treatments shown were begun. At zero time, the hydrocortisone-containing medium (preinduction medium) was decanted, the monolayers washed with HBSS, and medium containing the treatments shown placed on the monolayer cultures. For measurements of the rate of enzyme synthesis, 5.0 μc of H^3-leucine/-ml was added 15 min before collection of cells. Analyses were carried out as indicated in *Materials and Methods*. The rate of enzyme synthesis is expressed as relative radioactivity, which is defined as cpm in the isolated transaminase $\times$ 10^3/cpm per mg total soluble protein.

The observation that the induced enzyme level increased while the rate of enzyme synthesis decreased following actinomycin D exposure suggested that this antibiotic might be inhibiting processes involved in enzyme degradation. Figure 2 depicts the results of an experiment designed to examine this possibility. As in the previous experiment (Fig. 1), the enzyme level was elevated following actinomycin D addition to preinduced cultures. Actinomycin D had little or no effect on "chase" of H^3-leucine from the total prelabeled soluble proteins. Actinomycin D markedly inhibited the "chase" of H^3 from the prelabeled enzyme, whereas in cells not exposed to this inhibitor, the enzyme was found to undergo turnover with a half life of about three hours. Thus the elevation of enzyme activity following actinomycin D treatment of induced cells is the resultant of differential inhibitory effects on the rate of enzyme synthesis and degradation (cf. Figs. 1 and 2). As would be predicted, the enzyme level rises as a consequence of continued synthesis (although at a steadily lower rate) coupled with a marked and apparently immediate inhibition of degradation.

To ascertain whether this effect of actinomycin D was unique to the Reuber H-35 hepatoma, the HTC cell line was employed to carry out similar experiments. Figure 3 illustrates the time course of actinomycin D effects on the enzyme level and the rates of enzyme synthesis and degradation in preinduced HTC cells in

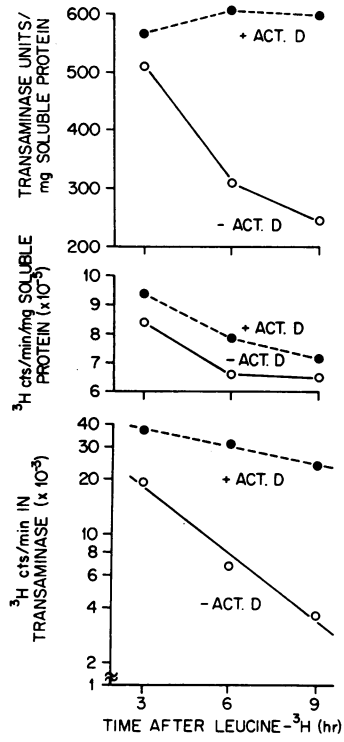

FIG. 2

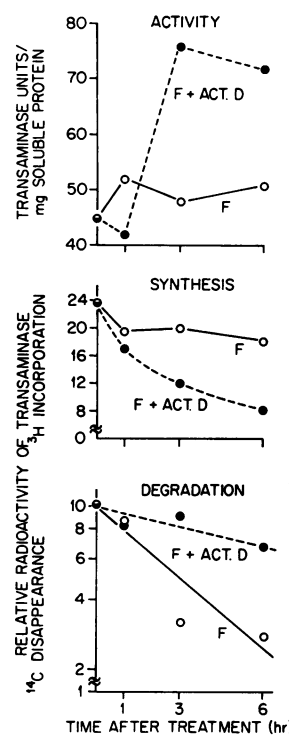

FIG. 3

FIG. 2.—Effect of actinomycin D on transaminase degradation in preinduced Reuber H-35 cultures in stationary phase. Hydrocortisone (10^{-6} *M*) was added to monolayer cultures 24 hr before, and H^3-leucine (5.0 μc/ml) was present during the final hour of this preinduction period. At the end of this 1-hr labeling period, the "H^3 medium" was decanted, the monolayers were washed with HBSS, and fresh medium was added without actinomycin D (*open circles*) or together with 5 μg of actinomycin D/ml (*solid circles*). Analyses were performed as described in *Materials and Methods*.

FIG. 3.—Effect of actinomycin D on transaminase synthesis and degradation in preinduced HTC cultures in stationary phase. Hydrocortisone (10^{-6} *M*) was added to monolayer cultures 24 hr before and C^{14}-leucine (0.5 μc/ml) 1 hr before these measurements were begun. Immediately preceding the zero time the "C^{14} medium" was decanted, the monolayers were washed with HBSS, and at zero time fresh medium was added containing hydrocortisone (10^{-6} *M*) alone (*open circles*) or together with 5 μg of actinomycin D/ml (*solid circles*). For rate of synthesis measurements, 7.5 μc of H^3-leucine/ml was added 15 min before collection of cells for analyses. Analyses were carried out as described in *Materials and Methods*.

monolayer culture. In this experiment, a dual isotope technique was employed to permit simultaneous measurement of both synthesis and degradation. The induced level of tyrosine transaminase in the HTC cell line is considerably lower than in the Reuber H-35 cells; however, the extent of hormonal induction in these two cell lines is quite similar (8- to 10-fold). The difference in the induced level of the enzyme is probably attributable to the fact that the basal enzyme level is markedly lower in HTC as compared with Reuber H-35 cells. Hydrocortisone plus actinomycin D brought about a maximum elevation of enzyme

activity by three hours, and this persisted for an additional three hours. During this interval, the rate of enzyme synthesis fell exponentially, again implying a functional half life of three hours for the transaminase mRNA. As in Reuber H-35 cells, the elevation of enzyme activity was explicable by the finding that the rate of enzyme degradation was rapidly and markedly inhibited by actinomycin D. The half life of transaminase following actinomycin D was 11.7 hours as compared with 3.0 hours in the control cells.

From the theoretical considerations set forth by Berlin and Schimke,[16] it would be predicted that the differential inhibitory effect of actinomycin D on the rates of transaminase synthesis and degradation would result in the observed elevation of the enzyme level (Fig. 3). At steady state, the enzyme level (E_{ss}) is equal to the rate constant of synthesis (k_1) over the rate constant of degradation (k_2), i.e.,

$$E_{ss} = k_1/k_2.$$

Therefore, if steady-state conditions are assumed for both the initial preinduced enzyme level and the approximate plateau level after actinomycin D, the relative change in any one of these three parameters should be predictable from the observed changes in the other two. Using the foregoing steady-state equation, the experimental data of Figure 3 have been found to conform well with theoretical results. This is demonstrated as follows: the preinduced enzyme level changed from 45 to 73 by 4.5 hours after actinomycin D, whereas the *relative* rate of enzyme synthesis decreased from 23.8 to 10 during this time. In order to calculate the expected *relative* change in the rate constant of degradation (k_2), the following steady-state equations may be written:

$$\text{at } t = 0 \text{ hours}, k_2 = \frac{23.8}{45} = 0.528, \quad (1)$$

$$\text{at } t = 4.5 \text{ hours}, k_2' = \frac{10}{73} = 0.137, \quad (2)$$

thus

$$k_2'/k_2 = \frac{0.137}{0.528} = 0.26.$$

It is apparent that the changes in the enzyme level and in the rate of synthesis at 4.5 hours after actinomycin D require that the rate of degradation (k_2) be decreased to one fourth of its initial value. The least-squares calculation of k_2 from the control curve and of k_2' from the data for the actinomycin-treated cells gave 0.100 hour^{-1} and 0.026 hour^{-1}, respectively. Thus the relative change in the experimentally determined k_2 agreed with the theoretical result.

Discussion.—The steady-state level of tyrosine transaminase (and other enzyme proteins) represents a balance between continual synthesis and degradation. Mechanisms for changing this level are now known to involve alterations in the rate of both synthesis and degradation. Data previously presented[17, 18] have shown that tyrosine transaminase induction by glucocorticoids in hepatoma

cell culture is the result of an increased rate of enzyme synthesis. However, the rise in tyrosine transaminase levels which follows the addition of actinomycin D to preinduced hepatoma cultures is primarily the result of a marked decrease in the rate of enzyme degradation. Synthesis of the enzyme is also inhibited by the antibiotic, but relatively slowly. Thus there occurs a period of time during which synthesis proceeds at a moderate rate while degradation is severely impaired, and as a consequence the enzyme level rises.

These findings are not in accord with those of Tomkins *et al.*,[19] who reported that actinomycin D increased the rate of transaminase synthesis after addition to preinduced HTC cultures. The discrepancy between these results and ours probably results from the fact that these investigators employed a 6.5-hour labeling period in the presence of actinomycin D in order to determine the rate of transaminase synthesis; the effects of actinomycin D on enzyme degradation were not studied. It is obvious from the half-life data we have presented that an interval of 6.5 hours encompasses approximately two half lives of the enzyme. During this interval, labeling of the enzyme can be increased due to the labeled amino acid's being incorporated into the enzyme and then retained as a result of blockage of degradation by actinomycin D.

Earlier results from our laboratory have demonstrated that inhibitors of protein synthesis (cycloheximide, puromycin) also interfere with tyrosine transaminase degradation.[3] It was suggested that certain protein(s) which turn over rapidly might be required for the degradative process. The results presented here further require, if this interpretation is to be maintained, that the half lives of the mRNA's for these degradative protein(s) be extremely short. A more likely explanation for the effects of inhibitors of protein and RNA synthesis can be proposed on the basis of the results of Mandelstam.[20] He suggested that inhibition by chloramphenicol of protein degradation in *E. coli* cultures in stationary phase results from the accumulation of compounds of small molecular weight that inhibit the degradative process. A similar mechanism may well be operative in the present case.

Summary.—With an isotopic-immunochemical procedure, the "superinduction" of tyrosine transaminase which occurs in preinduced hepatoma cells following the addition of actinomycin D was shown to be due to a differential inhibitory effect on the rate of transaminase synthesis and degradation. Following the addition of actinomycin D, the transaminase level rises as a result of a marked decrease in the rate of enzyme degradation combined with a steadily decreasing rate of synthesis.

We are pleased to acknowledge the kind assistance of Dr. Alfred Hellman, who tested our tissue cultures for bacterial and PPLO contamination, and Mrs. Joan Reel, who aided in the routine farming of the cultures. Special thanks are also due to Mr. Joseph Kendrick and Mr. Larry Roberson for their helpful assistance in certain phases of these experiments, and to Dr. Carl Wust for his aid in the preparation of antiserum. The cell lines used in this study were made available to us through the courtesy of Drs. Paul Morse and Van R. Potter (H35) and Drs. E. Brad Thompson and Gordon M. Tomkins (HTC).

* This research was supported jointly by the National Cancer Institute and by the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

† American Cancer Society postdoctoral fellow.

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