TURNOVER OF TYROSINE TRANSAMINASE IN CULTURED HEPATOMA CELLS AFTER INHIBITION OF PROTEIN SYNTHESIS[1]

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SUMMARY—Addition of cycloheximide to hydrocortisone-induced cell cultures of the Reuber (H-35) hepatoma results in an immediate decrease in the levels of tyrosine transaminase (L-tyrosine-2-oxoglutarate aminotransferase, E.C.2.6.1.5). The half-life of the enzyme is about 1.6 hr during the first hour after treatment; however, the rate of inactivation decreases such that the enzyme has a half-life of about 10 hr between 4 and 9 hr after addition of the inhibitor. The cells are functionally viable after 3 hr treatment with cycloheximide and retain their capacity both to inactivate tyrosine transaminase and to have the synthesis of this enzyme reinduced by hydrocortisone after removal of the inhibitor. The loss of ^{14}C-labeled tyrosine transaminase in H-35 cells is blocked by cycloheximide after a 1-hr period of normal turnover. Puromycin also blocks the turnover of the enzyme in the interval between 4 and 6 hr after addition of the inhibitor.

Tyrosine transaminase (L-tyrosine-2-oxoglutarate aminotransferase, E.C.2.6.1.5) is present in both rat liver and certain rat hepatoma cell lines (1–3). The amount of this enzyme in livers of adrenalectomized rats and in cultures of two hepatoma cell lines can be elevated nearly 10-fold by hydrocortisone administration (1–4). The steroid-mediated increase in this enzyme is caused by an enhanced rate of enzyme synthesis rather than a decreased rate of enzyme inactivation (5–6). Inhibition of enzyme synthesis by administration of cycloheximide to adrenalectomized rats results in the simultaneous blockage of enzyme degradation, as indicated both by the failure of enzyme levels to decline and by the maintenance of the relative

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specific radioactivity of the enzyme at a constant level during the interval of treatment (7). Administration of other protein synthesis inhibitors (puromycin, azaguanine, and azacytidine) yields results similar to those with cycloheximide (8–9). In this report we show that cycloheximide and puromycin will also block tyrosine transaminase turnover in stationary-phase, hydrocortisone-induced Reuber (H-35) hepatoma cells, and that the processes of tyrosine transaminase induction and enzyme turnover are able to resume after removal of the inhibitor.

MATERIALS AND METHODS

Reuber (H-35) cells were grown to monolayers at 37°C in plastic tissue culture flasks (Falcon, 76 cm^2). The growth medium was Eagle's basal medium enriched fourfold with amino acids and vitamins and supplemented with 20% fetal calf serum and 5% calf serum. All experiments were performed with early stationary phase (8- to 9-day) cultures in serum-free, unenriched medium. Tissue culture materials were purchased from Grand Island Biological Company.

The characteristics of this cell line under these conditions, preparation of cell extracts, assay of tyrosine transaminase activity and immunochemical analysis of enzyme turnover, are as described by Reel and Kenney (4). Cycloheximide and puromycin were obtained from Sigma Chemical Company and Nutritional Biochemicals Company, respectively.

RESULTS

The effectiveness of increasing doses of cycloheximide in inhibition of incorporation of ^{3}H-leucine into total soluble proteins in uninduced H-35 cells over a 6-hr interval is indicated in Table 1. A dose of 5 μg/ml was found to inhibit the rate of incorporation of ^{3}H-leucine during a 30-min pulse into total soluble protein by >95% and was equally effective when protein synthesis was measured at 30 min or 5-1/2 hr after addition of the antibiotic. This dose was chosen for use in subsequent experiments to assure maximal inhibition of protein synthesis throughout the course of these studies, with a minimum of possible side effects caused by much larger doses.

Addition of 5 μg/ml of cycloheximide to H-35 cells induced maximally with 5×10^{-7} M hydrocortisone results in an initial decline in enzyme levels

TABLE 1

Effect of Increasing Amounts of Cycloheximide on the Rate of Incorporation of ^{3}H-Leucine into the Total Soluble Proteins of H-35 Cells

Monolayers were maintained for 20 hr with serum-free medium prior to addition of cycloheximide at zero time. At 30 or 330 min, 0.5 μCi/ml of ^{3}H-4,5-leucine (5.03 Ci/mole) was added, and the cells were harvested 30 min later. Cells were disrupted in 0.15 M KCl - 0.001 M EDTA (pH 8.0) by freezing and thawing 3 times in liquid nitrogen. Specific radioactivity of the 105,000 g supernatant proteins was determined using the filter paper disc technique for radioactivity (11) and the Lowry technique (12) for proteins.

Cycloheximide added	Radioactivity of total soluble protein Pulse period			
	30 – 60 min		330 — 360 min	
μg/ml	cpm/mg	%	cpm/mg	%
0	13,385	100	54,368	406
2	702	5.2	1,718	12.8
5	573	4.2	629	4.6
10	388	2.8	606	4.5
20	334	2.4	250	1.8

during the first hour ($t_{1/2}$ = 1.6 hr). The rate of enzyme loss decreases after this interval and a half-life of 10.5 hr is observed throughout the interval between 4 and 9 hr after addition of the inhibitor (Fig. 1). Wash-away experiments were conducted on a series of similarly treated monolayer cultures 3 hr after addition of cycloheximide. Within 1 hr after removal of the inhibitor, the levels of tyrosine transaminase increase to a level that is 96% of that present in maximally induced uninhibited cells. If hydrocortisone is not added back to the cultures after cycloheximide is removed, the cells regain their capacity to inactivate the enzyme, and its activity decreases with the characteristic half-life of 1.5 hr, which continues until the enzyme reaches its basal steady-state level. If hydrocortisone is added back after cycloheximide removal, reinduction of tyrosine transaminase synthesis occurs with a characteristic 1-hr lag and at a rate that is identical to that observed after addition of hydrocortisone to uninduced cultures. If cycloheximide is added back to the washed

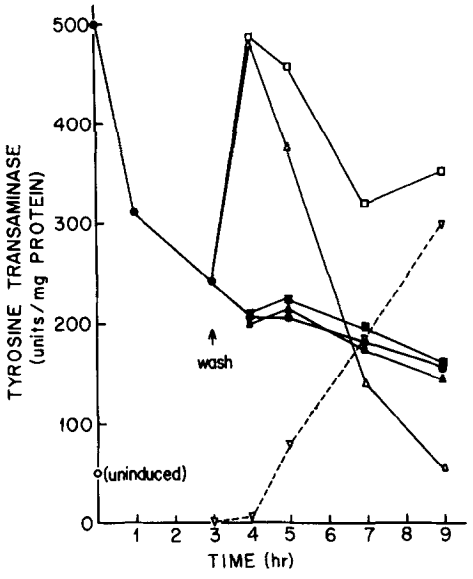

FIG. 1. Effect of cycloheximide on the turnover of tyrosine transaminase in hydrocortisone-induced H-35 cells and the restoration of both hormone inducibility and enzyme turnover after removal of the inhibitor. Monolayers were maintained for 20 hr with serum-free medium containing hydrocortisone (5×10^{-7} M). Cycloheximide (5 μg/ml) was added and enzyme activity was measured at the indicated times without further treatment (●). Four groups of cells were washed 3 times with fresh medium 3 hr after addition of the inhibitor, and the following additions were made: no additions (△), 5×10^{-7} M hydrocortisone (□), 5 μg/ml cycloheximide (▲), or 5×10^{-7} M hydrocortisone plus 5 μg/ml cycloheximide (■). Postwash hydrocortisone inducibility (▽) is the difference between the group with no additions and the group which received only hydrocortisone. Values represent the average of two identically treated flasks of cells.

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cultures (with or without adding back hydrocortisone), the rate of enzyme decline is identical to that observed in the unwashed, inhibited cultures (Fig. 1).

Direct measurements of the effect of cycloheximide on the rate of tyrosine transaminase degradation were made, employing immunochemical analysis for measurement of the rate of loss of ^{14}C-labeled tyrosine transaminase in hydrocortisone-preinduced monolayer cultures in the presence and absence of 5 μg/ml of cycloheximide (Fig. 2). These data confirm the conclusions made above from measurement of enzyme activity changes; i.e., tyrosine transaminase is degraded at a normal rate for 1 hr after addition of cycloheximide, after which its turnover is effectively blocked.

A comparison of the effects of puromycin and cycloheximide on the rate of

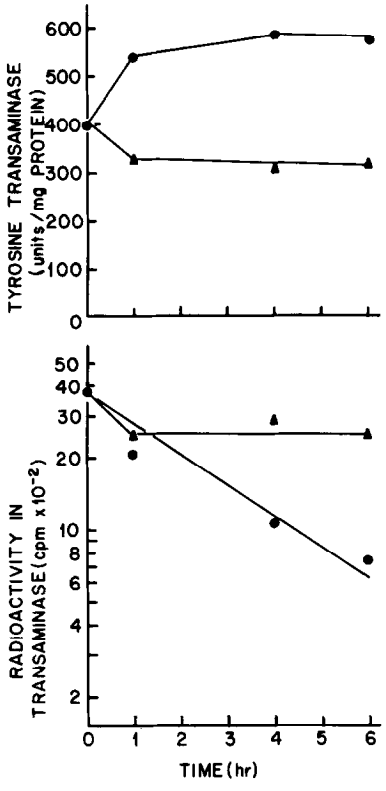

FIG. 2. Tyrosine transaminase degradation in control and cycloheximide-treated monolayers of H-35 cells. Monolayers were maintained for 12 hr in serum-free medium with hydrocortisone (5×10^{-7} M). ^{14}C-Leucine (0.5 μCi/ml, 316 mCi/mmole) was added to groups of 6 flasks. Two hr later the monolayers were washed with fresh medium containing hydrocortisone. Three groups of cells received 5 μg/ml of cycloheximide (▲) and four groups were maintained as controls (●). Cells were harvested at the indicated times after removal of the ^{14}C-leucine. Tyrosine transaminase was assayed, isolated by immunochemical precipitation and the total radioactivity in this enzyme was determined as described previously (4).

tyrosine transaminase turnover measured by immunochemical analysis of the loss of ^{14}C-labeled tyrosine transaminase during the interval between 4 and 6 hr after addition of the inhibitors is given in Table 2. It was determined that a dose of 200 μg/ml of puromycin is the minimum dose required to block the induction of tyrosine transaminase synthesis in H-35 monolayer cultures by hydrocortisone (unpublished observation). Turnover of the enzyme is effectively blocked by both puromycin and cycloheximide during this interval, while the uninhibited cells lose half of the labeled enzyme. The amount of radioactivity in the puromycin-inhibited cells is slightly higher than that in the cells treated with

TABLE 2

Effect of Cycloheximide and Puromycin on the Turnover of Tyrosine Transaminase in Hydrocortisone-Induced H-35 Cells

Cells were pretreated for 22 hr with serum-free medium containing hydrocortisone (5×10^{-7} M). ^{14}C-Leucine (0.5 μCi/ml, 316 mCi/mmole) was added to groups of six monolayer cultures. Two hr later the cells were washed three times with fresh medium, at which time two groups received no additions, two received cycloheximide (5 μg/ml), and two received puromycin (200 μg/ml). Groups of flasks from each treatment were harvested 4 and 6 hr later. Tyrosine transaminase was isolated by immunochemical precipitation, and the total radioactivity in this enzyme was determined as described previously (4).

Treatment	Tyrosine transaminase radioactivity	
	Time after treatment	
	4 hr	6 hr
None	2,813	1,426
Cycloheximide	3,547	3,683
Puromycin	4,314	4,074

cycloheximide, suggesting that its effect may be manifested earlier than that of cycloheximide.

DISCUSSION

These results confirm earlier observations made with the rat liver in vivo (7), where it was shown that agents which block synthesis of protein also block the intracellular degradation of hepatic tyrosine transaminase. Unlike the liver system, the effects of cycloheximide on enzyme degradation in H-35 cells in culture are manifested only after a distinct lag of 1–2 hr. This difference may be apparent rather than real, for the extensive variation encountered in the earlier in vivo experiments could have obscured the lag (7). Indeed, Levitan and Webb have observed that azaguanine and azacytidine administered to rats in vivo will block degradation of tyrosine transaminase, but only after a period of about 2 hr, during which degradation proceeds at more or less the normal rate (13,14). Thus the available data are consistent with the view that a cellular component with an effective lifetime of 1–2 hr

in both rat liver and cultured hepatoma cells is required for transaminase degradation; as suggested before, this labile component may be a specific polypeptide involved as catalyst or reactant in degradation of tyrosine transaminase (7). Alternatively, it may be that cessation of protein synthesis results in the accumulation of a substance which blocks transaminase degradation (4).

The data of Fig. 1 show clearly that the effects of cycloheximide on transaminase synthesis and degradation are reversible, and they render unlikely the possibility that inhibition of degradation reflects a generalized toxic effect on the cultured cells. The rapid rebound of the enzyme to fully induced levels after cycloheximide removal is especially noteworthy. This indicates that the hydrocortisone-induced level of transaminase messenger remains essentially constant despite inhibition of translation, and also that the potential translation rate is much greater than that usually observed (cf. synthesis of 240 units/hr after cycloheximide removal to the maximal rate of 90 units/hr during hydrocortisone induction). This rapid translation appears to have as a consequence a rapid disappearance of messenger, for induced synthesis ceases completely 1 hr after cycloheximide removal. If the messenger retained its usual stability ($t_{1/2}$ about 2 hr, ref. 10) under these conditions, induced synthesis should persist for several hours without inducer; and in cells resupplemented with the inducer, synthesis should continue without delay.

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