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Evidence for Reversible Inactivation of Induced Tyrosine Aminotransferase in Rat Liver *In Vivo*

(cortisol/puromycin/Sprague–Dawley rats)

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ABSTRACT **Induction of tyrosine aminotransferase (EC 2.6.1.5) with cortisol is followed by a rapid phase of inactivation of the enzyme, during which administration of puromycin causes a sharp *increase* in enzyme activity. The increase is not due to puromycin-resistant synthesis of protein, since precipitation of the enzyme with antibodies to tyrosine aminotransferase after pulse labeling inhibited incorporation of radioactive amino acids. Reactivation of the enzyme was specific, since the structurally similar dimethylaminopurine and purine aminonucleoside were ineffective. An action of puromycin other than its capacity to inhibit protein synthesis was required for reactivation, since cycloheximide did not demonstrate such an effect. *In vitro* studies with free and bound polysomes, isolated during the phase of inactivation, indicated that reactivation by puromycin was not due to release of nascent tyrosine aminotransferase peptides. We conclude that the enzyme is initially reversibly inactivated; therefore, the overall degradation of this intracellular protein is a multistep phenomenon.**

Cells from higher organisms contain a characteristic quantity of protein, most of which is in a continual state of turnover (1-3) at rates varying with individual proteins (4). For example, one-half the ornithine decarboxylase in rat liver is replenished about every 11 min (5), while about 4-5 days are required for one-half the arginase to be degraded and resynthesized (6). The mean turnover, or half-time (t $^1/_2$), of soluble liver protein is about 3 days (7, 8). Since liver cells divide infrequently, as evidenced by only one mitosis per 10,000-20,000 cells observed (9), the average generation time of the rat liver hepatocyte has been estimated to be several months to 1 year. Therefore, soluble liver protein is degraded and synthesized many times over the lifetime of a given cell.

For prevention of unusual deviations in concentration of these proteins there must be integrated control of both the synthesizing and degrading mechanisms; that is, the net change in enzyme concentration over a given period should approach zero. One method for such coordination is by a constant input of enzyme (zero order synthesis) and an output proportional to the concentration of the protein being synthesized (first order degradation). In this type of control the concentration of enzyme can be regulated, without the mediation of complicated feedback mechanisms, by some device sensitive only to concentration of enzyme. A consequence of such a mechanism is that induction or suppression of enzyme activity may occur not only as a result of alteration of the rate of enzymatic synthesis, but also by adjustment of the rate of degradation. In fact, the most efficient way to induce an enzyme would be by inhibition of enzyme degradation and not by stimulation of enzyme synthesis. However, for enzymes with a slow rate of turnover, this mechanism would not allow for rapid changes in enzyme activity. Increasing synthesis alone, on the other hand, is uneconomical since an increase in the number of enzyme molecules would of necessity increase enzyme degradation. For economy and rapidity, inducers of *de novo* enzyme synthesis should also inhibit enzyme degradation. We have recently demonstrated this for induction of tyrosine aminotransferase (EC 2.6.1.5) (TA) by cortisol (8), and this is probably not an isolated instance.

Clearly, enzyme degradation should play a prime role in regulation of enzyme activity, but there is little, if any, information regarding the mechanism(s) of intracellular protein degradation. The present work has provided such information for TA.

MATERIALS AND METHODS

Animals. Adrenalectomized male rats of the Sprague-Dawley strain (2-3 months old) weighing about 200 g, from Charles River Laboratories (Wilmington, Mass.), were maintained for at least 2 days after arrival on rat pellet diets supplemented with drinking water containing 0.9% NaCl.

Tissue Analysis. Each rat was anesthetized with pentobarbital (30 mg/kg, intraperitoneally); the upper abdominal cavity was exposed by a 2.5-cm (1-inch) midline incision. Biopsies were taken by cutting wedge-shaped samples from the edges of the liver lobes (10). The biopsy sample was weighed, homogenized in 1 ml of cold (4°) 0.1 M sodium phosphate buffer (pH 7.5) with glass tissue homogenizers, and centrifuged at about 18,000 × g at 4° for 20 min. The supernatant was assayed for TA (11) and protein (12).

Determination of Radioactivity Incorporated into TA. Highly purified TA, antibody preparations, and procedures for the various techniques used are as in (8). Additional details are indicated in legends of the figures.

In Vitro Experiments. Free and bound polysomes were prepared by the methods of Blobel and Potter (13, 14). Experiments on incorporation of L-[^{14}C]leucine and release of methoxy-[^{3}H]puromycin were performed according to the procedure of Blobel and Potter (13), and Blobel and Sabatini (15).

Abbreviation: TA, tyrosine aminotransferase.

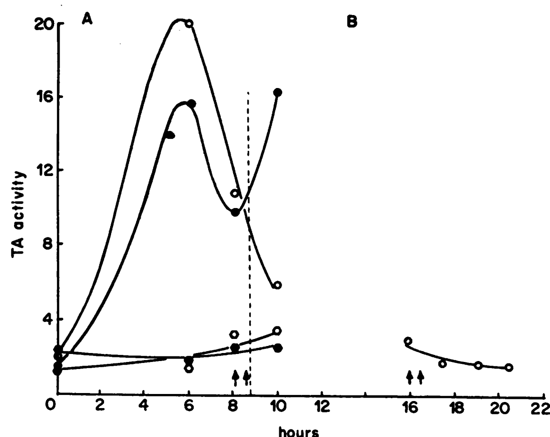

FIG. 1. Changes in activity of TA after injection of cortisol and puromycin. (*A*) Adrenalectomized male rats were anesthetized with pentobarbital (30 mg/kg of body weight); about 50 mg of the liver of each rat was removed. Rats were divided into 4 groups as indicated in text. Biopsy samples were weighed, homogenized in 1.0 ml of 0.1 M sodium phosphate (pH 7.5), and centrifuged for 20 min at 18,000 × *g*. The supernatants were assayed for TA activity, which is expressed as μmol of *p*-hydroxyphenylpyruvate formed/hr per 100 mg of tissue. At the times indicated by *arrows*, 5.0 mg of puromycin dihydrochloride (neutralized) was administered to the rats that were injected with cortisol (●——●) and saline (●——●). Curves denoted by (O——O) and (O——O) represent control groups of rats that were injected with hormone and saline, respectively. 80 min after intraperitoneal injection of 25 μCi L-[^{14}C]aminoacid mixture in all rats, the livers were removed and quickly frozen on dry ice. Further analysis was continued the following morning as indicated in Tables 1 and 2. (*B*) Two rats were injected intraperitoneally with 10 mg of cortisol. 16 hr later, the rats were anesthetized and liver biopsies were taken. Puromycin (5.0 mg) was administered (*arrows*) and additional biopsies were taken at 1.5-hr intervals. TA activity in each rat was almost identical to the average activities shown.

Puromycin dihydrochloride, puromycin aminonucleoside, and dimethylaminopurine were obtained from Nutritional Biochemical Co. (Cleveland, Ohio); L-[^{14}C]aminoacid mixture, L-[^{14}C]leucine (20 Ci/mol), and methoxy-[^{3}H]puromycin (1 Ci/mol) from New England Nuclear Corp. (Boston, Mass.).

RESULTS AND DISCUSSION

Fig. 1*A* illustrates the paradoxical effect of puromycin on TA activity after induction with cortisol. During the latter phase of the induction cycle a return of enzyme activity occurred soon after administration of puromycin (*solid circles*). To determine whether this reflected *de novo* synthesis resistant to puromycin, or reactivation of previously inactivated enzyme, pulse labeling with a mixture of L-[^{14}C]aminoacids was performed. Two of four groups of rats were injected with saline, and the other two groups were injected with 10 mg of cortisol/kg of body weight, intraperitoneally. After 8 hr, each rat in one of the groups injected with saline and in one of the groups inoculated with cortisol, was injected with 5 mg of puromycin, intraperitoneally; this was repeated 0.5 hr later. After 10 min, all four groups were injected with 25 μCi of the mixture of L-[^{14}C]aminoacids intraperitoneally (*vertical dashed line* of Fig. 1*A*). 80 min later, the livers were removed and the radioactivity incorporated into total protein and into TA was determined.

The radioactivity incorporated into TA for each group of rats is shown in Table 1. Counts obtained after the second precipitation with antibody are considered to be derived from nonspecific antibody–protein interactions (16), since a third precipitation yielded the same number of counts (17). The reduced radioactivity observed after treatment with puromycin indicates that the marked increase in enzyme activity could not have been due to *de novo* synthesis, especially as the increase in activity occurred while overall synthesis of protein and TA were being inhibited, the protein by about 70% in rats treated with saline and about 80% in rats treated with cortisol; TA was inhibited about 50 and 87%, respectively (Table 2). Moreover, in agreement with previous findings (18), synthesis of TA returns to control levels (261 cpm/100 mg protein) during the latter phase of the induction cycle (Table 2).

The capacity of puromycin to reactivate TA is limited to a finite period of the induction cycle. There was no reactivation

TABLE 1. *Precipitation of radioactive TA with specific antibodies in control and puromycin-treated rats*

Treatment	1st ppt	2nd ppt	1st − 2nd	Protein (mg/ml of tissue extract)	cpm/ 100 mg Protein
	(counts per 20 min/ml of tissue extract)				
Saline	4040	1150	2890	45.6	317
	2770	775	1990	38.4	259
	2740	849	1890	45.8	206
Saline + puromycin	1500	669	827	41.6	98
	1910	751	1160	44.2	131
	2150	803	1350	41.2	164
Cortisol	2950	633	2320	43.6	265
	2840	823	2010	36.2	278
Cortisol + puromycin	737	599	138	39.6	19
	909	545	354	40.4	44
	1130	735	395	40.0	49
	1040	745	293	39.6	37

Frozen livers from the experiments of Fig. 1 were thawed. About 3 g were homogenized in 6 ml 0.1 M sodium phosphate (pH 7.5) containing 2-oxoglutarate, 1 mM; pyridoxal phosphate, 0.1 mM; and ethylenediamine tetraacetic acid (EDTA), 0.1 mM; centrifuged at 15,000 × *g* for 30 min and again at 100,000 × *g* for 1 hr. 3-ml Aliquots were heated at 57° for 3 min, immersed in a salt water bath at about −10° for 30 min, and centrifuged. TA activity was intact in the supernatant. To 1.0-ml aliquots of the supernatant fractions, sufficient purified nonradioactive TA was added to bring the level of enzyme activity up to that contained in the most active fraction. Volume was adjusted to 1.5 ml with the above buffer and each fraction was precipitated with 20% excess antibody to TA. After 1 hr at 37° and then overnight at 4°, there was no detectable enzyme activity. Precipitated enzyme was recovered by centrifugation, washed 3 times with 1 ml of 0.1 M sodium phosphate in saline (pH 7.2), dissolved in 0.2 ml of 0.1 N KOH, and quantitatively transferred to counting vials [1st precipitate (ppt)]. Purified nonradioactive enzyme was again added to the supernatants of the first precipitation and the above procedure was performed again to obtain a second antibody precipitate. Radioactivity in the 1st ppt minus that in the 2nd ppt represents radioactivity in TA.

of enzyme activity 16 hr after administration of cortisol (Fig. 1*B*). The earlier reactivation of TA appears to be dependent on the entire structure of the molecule, since neither dimethylaminopurine nor puromycin aminonucleoside reactivated the enzyme in doses that have no effect on synthesis of mouse liver proteins (19). Also, inhibition of protein synthesis by cycloheximide was not accompanied by reactivation of the enzyme, although it prevented further inactivation (Fig. 2).

Puromycin acts as a peptide-tRNA acceptor, causing formation and release of puromycyl-peptides (21–23), while cycloheximide appears to inhibit initiation and elongation of polypeptide chains (24, 25). It seemed possible that the apparent reactivation of TA by puromycin might be merely release of nascent enzyme chains present on polysomes. Free and bound polysomes, shown in a control experiment to be functional, were accordingly isolated from rat livers during the rapid phase of enzyme synthesis (3 hr after administration of cortisol) and during the inactivation phase of the induction cycle (9 hr after administration of cortisol). The data in Table 3 indicate that methoxy-[^{3}H]puromycin was incorporated into Cl_3CCOOH-precipitable material during incubation with free polysomes at 37°. Little incorporation was noted with bound polysomes. No difference in methoxy-[^{3}H]puromycin uptake or increment in TA was observed between free polysomes isolated 3 or 9 hr after administration of cortisol. Also, aliquots of both the 0 and 37° incubation mixtures that were made 0.5 M with respect to KCl and were incubated an additional 15 min at 0° demonstrated no increase in enzyme activity. This concentration of KCl did not affect enzyme activity in control assays. The data in Table 4 indicate significant incorporation of L-[^{14}C]leucine that was sensitive to puromycin. As above, there was no difference in enzyme activity before or after incubation, or between the puro-

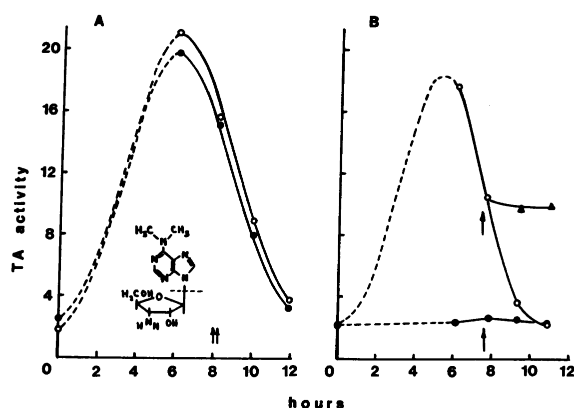

FIG. 2. Changes in TA activity after administration of cortisol, dimethylaminopurine, puromycin aminonucleoside, and cycloheximide. (*A*) Six rats were anesthetized with pentobarbital (30 mg/kg of body weight) and liver biopsy samples were taken. Cortisol (10 mg) was administered (zero time) and additional biopsies were taken 6 and 8 hr later. At the times indicated by *arrows*, half the animals were injected with 3 mg of puromycin aminonucleoside (formula shown ●——●), and the other half with 2 mg of dimethylaminopurine (upper half of formula shown ○——○). Doses were adjusted so that the molar concentration was about the same as for puromycin (Fig. 1). Points represent average TA activities expressed as μmol of *p*-hydroxyphenylpyruvate formed/hr per 100 mg of liver. All injections were intraperitoneal. (*B*) Rats were anesthetized and their liver biopsies were removed as indicated above. Cortisol was administered to 5 rats and saline was administered to 2 rats. Biopsy samples were taken 6 hr later. 7.5 hr After administration of cortisol, cycloheximide (100 μg/100 g of body weight) was injected into 3 rats that were treated with cortisol (▲——▲) and into two rats that were treated with saline (●——●), as indicated by the *arrows*. The two other rats that were treated with cortisol were injected with saline (○——○). Additional liver biopsies were taken as indicated. Each point represents the average TA activity for each group of animals.

TABLE 2. *Effects of puromycin on incorporation of* L-[^{14}*C*]*aminoacids into total protein and TA*

Treatment	Cl_3CCOOH soluble (cpm/100 mg of protein)	Cl_3CCOOH insoluble (cpm/100 mg of protein)	TA activity (μmol/hr per 100 mg of protein)	TA (cpm per 100 mg of protein)
Saline (3)	55,800	99,300	15.9	261
Saline + puromycin (3)	123,000	31,500	9.3	131
Cortisol (2)	76,300	124,000	25.0	272
Cortisol + puromycin (4)	116,000	26,200	122.0	37

Aliquots (0.2 ml) of the 100,000 × *g* supernatant, prepared as described in Table 1, were precipitated with an equal volume of 10% Cl_3CCOOH and left at 4° overnight, and centrifuged; an aliquot of the supernatant was set aside for counting (Cl_3CCOOH soluble counts). The precipitate was washed 3 times with 5 ml of 5% Cl_3CCOOH, dissolved in 0.2 ml of 0.1 N KOH, and counted. Average values are given for the number of animals in parentheses. Enzyme activity is expressed as μmol of *p*-hydroxyphenylpyruvate formed.

TABLE 3. *Incorporation of methoxy-*[^{3}*H*]*puromycin into nascent peptides and release of TA from free and bound polysomes of rat livers 3 and 9 hr after injection of cortisol in vivo*

Time after cortisol (hr)	°C	cpm	Ribosomal protein (mg)	Ribosomal protein (cpm/mg)
		Free polysomes		
3	0	107	2.96	36
	37	350	2.96	118
9	0	58	3.26	18
	37	345	3.26	106
		Bound polysomes		
3	0	47	1.82	26
	37	68	1.82	37
9	0	68	2.07	33
	37	118	2.07	57

Free and bound polysomes were isolated from the livers as in (14). Polysome pellets were suspended in 6.0 ml of ice-cold Tris buffer (pH 7.5 50 mM; $MgCl_2$, 5 mM; KCl, 25 mM; and sucrose, 250 mM). To 0.9 ml of suspension was added 0.1 ml methoxy-[^{3}H]puromycin (10 μCi/ml, 1 Ci/mol). After 10 min at 0° or 37°, 0.2 ml was taken for TA assay and to the remainder, 0.8 ml of 10% Cl_3CCOOH was added. The precipitate was centrifuged, washed 3 times with 5 ml of 5% Cl_3CCOOH, dissolved in 0.2 ml of 0.1 N KOH, and counted. TA activity was not changed.

TABLE 4. *Release of TA in an in vitro protein synthesizing system by free polysomes isolated during induction (3 hr) and inactivation (9 hr) phases after administration of cortisol*

	3 hr	9 hr
	(cpm/mg of ribosomal protein)	
37°	4430	3620
ΔTA activity*	No change (NC)	NC
0°	1400	1440
ΔTA activity	NC	NC
37° + puromycin	2000	1790
ΔTA activity	NC	NC

Free polysomes prepared as in Table 3, were incubated 40 min in 1 ml containing ATP, 4.5 μmol; GTP, 1 μmol; creatine phosphate, 10 μmol; creatine phosphokinase, 5 μg; 100,000 × *g* supernatant fraction from control animals (untreated with hormone) that had been passed through Sephadex G-75, 0.25 ml; 19 amino-acid mix minus L-leucine, 25 nmol each; L-[^{14}C]leucine (20 Ci/mol), 1 μCi; Tris buffer (pH 7.5), 50 μmol; $MgCl_2$, 5 μmol; KCl, 25 μmol; and where indicated, puromycin, 1 μmol. 0.2 ml was taken for TA assay, the remainder was acidified with 0.8 ml of 10% Cl_3CCOOH and heated at 90° for 15 min. The precipitated polypeptides were washed 3 times with 5% Cl_3CCOOH, dissolved in 0.2 ml of 0.1 N KOH, and counted.

* Change in tyrosine aminotransferase activity.

mycin-treated and untreated incubation mixtures. Therefore, it is unlikely that release of enzyme from polysomes is responsible for enzyme reactivation *in vivo*.

The present data indicate that active tyrosine aminotransferase (E_A) is initially converted to an inactive form (E_I), which is not degraded immediately since reactivation by puromycin to (E_A) can occur over a period of several hours (10). Complete conversion of (E_I) to an irreversibly inactivated (e_d) form occurs, however, by the time enzyme activity returns to near basal levels (Fig. 1*B*), since puromycin does not then reactivate the enzyme. Therefore, the major conclusion we infer is that the intracellular degradation process of TA is complex and involves more than a single-step reaction, such as:

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