Appearance of an Increment of Tyrosine Aminotransferase Activity in a Cell-free System*

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The appearance of tyrosine aminotransferase activity during incubation of cell-free systems obtained from rat liver is described. Cell-free systems from normal rat liver develop tyrosine aminotransferase activity in a nearly linear fashion during 90 minutes of incubation of a microsomal-supernatant system under nitrogen. The extent of the appearance of enzymatic activity during this incubation period was in the range of 50–130% over the zero-time controls. The results of enzyme assays are supported by radioactivity measurements of the 2,4-dinitrophenylhydrazone of [^{14}C]-*p*-hydroxyphenylpyruvate formed from L-[U-^{14}C]tyrosine. The increase in enzymatic activity of microsomal-supernatant systems is dependent on the temperature and *p*H of incubation and is stimulated by DPN$^+$. Appearance of enzymatic activity is also slightly stimulated (59%) by 0.03 mM 3′,5′-cyclic-AMP. Appearance of enzymatic activity is only slightly affected by inhibitors of protein synthesis (puromycin and ethionine), and is relatively immune to added RNAase or nuclear RNA preparations. Possible explanations for the incubation-dependent appearance of enzymatic activity in these systems are given.

It was recently reported from our laboratory that liver preparations from adrenalectomized rats showed increases to the extent of 10% in tyrosine aminotransferase activity after a short incubation period (Diamondstone and Litwack, 1963a). Whereas the apparent increase in enzymatic activity was stimulated by addition of DPN$^+$, addition of hydrocortisone and tyrosine either inhibited or had no effect. A major problem was the difficulty in obtaining consistently reproducible experimental results. Conditions have been found whereby the reproducibility of results is more satisfying. In this communication are described the properties of a cell-free system obtained from normal rats and the effects of various agents on the development of the enzymatic activity in this system.

EXPERIMENTAL

Subcellular preparations were obtained from liver homogenates of normal male Wistar rats (Huntington Farms, Inc., Phila., Pa. or Carworth Farms, Inc., New York City, N. Y.). These consisted either of microsomes plus supernatant or of mitochondria, microsomes, and supernatant.

Fresh chilled rat liver was minced and homogenized in a Potter-Elvehjem homogenizer with 8 ml of chilled medium per g liver (Diamondstone and Litwack, 1963a). The homogenate was centrifuged at 600 × *g* at 2° for 30 minutes and the supernatant fraction thus obtained was the mitochondrial-microsomal-supernatant. This fraction contained, on the average, 1.41 mg Kjeldahl nitrogen per ml. The microsomal-supernatant fraction was obtained by further centrifuging the mitochondrial-microsomal-supernatant at 15,000 × *g* for 15 minutes. The microsomal-supernatant contained, on the average, 0.74 mg Kjeldahl nitrogen per ml.

The complete incubation system is essentially that of Lingrel and Webster (1961), as modified by Diamondstone and Litwack (1963a). Unless otherwise specified, it consisted of the following: 6 ml of the mitochondrial-microsomal-supernatant; 2.3 ml of 0.1 M Tris-HCl, *p*H 7.8, containing 0.1 M KCl; 0.1 ml of 0.1 M magnesium chloride; and 10 μmoles ATP, 10 μmoles DPN$^+$, 90 μmoles α-ketoglutaric acid, 10 μmoles of crystalline manganese chloride, and 1 mmole glycine, all added in the solid form.

The system was incubated in air or under nitrogen at 37° in a gyrotory water-bath shaker and 1-ml aliquots were withdrawn at the beginning (zero time) and at subsequent intervals. Aliquots were immediately assayed for tyrosine aminotransferase activity, by adding these to an assay system containing 8.0 ml of 0.2 M sodium phosphate buffer, *p*H 7.2; 0.5 ml of a 300 μg/ml solution of pyridoxal phosphate; 0.5 ml of 0.3 M α-ketoglutarate; and 1.0 ml of 0.06 M sodium diethyldithiocarbamate (Lin and Knox, 1957). In later experiments, wherever indicated, all reagents in the assay system were reduced in volume by 80%, except that the volume of the aliquot(s) taken from the incubation system remained the same. The assay mixture was brought to 37° in the water-bath shaker. The reaction was started with the addition of 5 ml of 12 mM tyrosine in sodium phosphate buffer (0.2 M, *p*H 7.2) and stopped at the end of 20 minutes' incubation with the addition of 0.5 ml of 100% trichloroacetic acid. The resulting precipitates were centrifuged and 2.0 ml of the clear supernatants was taken for estimation of *p*-hydroxyphenylpyruvic acid by the Briggs method (Briggs, 1922), as modified by Canellakis and Cohen (1956), except that 2.0 ml of water and 4.0 ml of the "mixed reagent" were added to the 2.0 ml of the clear supernatant. The appearance of tyrosine aminotransferase activity in the incubation system is expressed as the difference in the number of micromoles of *p*-hydroxyphenylpyruvic acid produced during a 20-

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TABLE I

EFFECTS OF PUROMYCIN, ACTINOMYCIN D, OR DPN$^+$ ON THE APPEARANCE OF TYROSINE AMINOTRANSFERASE ACTIVITY *in Vitro*[a]

Inhibitor or Stimulator	No. of Expts	Concentration of Inhibitor or Stimulator (μmoles *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant per 90 min incubation)		
Puromycin·2 HCl	3	0	0.7×10^{-3} M	1.5×10^{-3} M
		0.80 (0.64–1.00)[b]	0.60 (0.44–0.67)[b]	0.50 (0.50–0.52)[b]
Actinomycin D	2	0	1.0×10^{-5} M	2.0×10^{-5} M
		0.54 (0.47–0.61)[b]	0.57 (0.44–0.70)[b]	0.50 (0.44–0.56)[b]
DPN$^+$	5	0	1.0 μmole/ml	
		0.40 (0.23–0.61)[b]	0.77 (0.35–1.71)[b] $P < 0.20$[c]	

[a] The incubation system contains the microsomal-supernatant. The incubation was carried out under nitrogen at 37° for 90 minutes. The value of enzymatic activity is derived from the 1.0-ml sample taken from the incubation system after 90 minutes and has been corrected by subtraction of the value of enzymatic activity derived from the 1.0-ml sample taken from the incubation system at zero time. The values presented are the average of experiments cited. Zero-time activity was 4.35 μmoles of *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant during a 20-minute enzymatic assay, or 5.87 μmoles of product formed per mg Kjeldahl nitrogen. [b] Indicates the range. [c] Statistical analysis was performed by comparing the values of the control with the values of the control plus DPN$^+$.

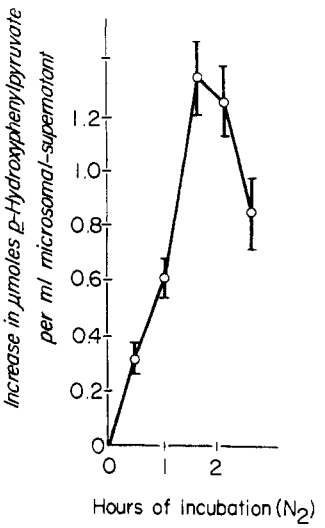

FIG. 1.—Rate of appearance of tyrosine aminotransferase with respect to incubation time. Incubation was carried at 37° and under nitrogen using a microsomal-supernatant. Each point is the average of three experiments. Transferase activity at zero time of incubation (activity without incubation) was 4.55 μmoles of *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant during a 20-minute enzymatic assay, or 6.15 μmoles of *p*-hydroxyphenylpyruvate formed per mg Kjeldahl nitrogen during a 20-minute enzymatic assay. The vertical bar at each point indicates the range of values.

minute-assay period per ml of the microsomal-supernatant between the beginning (zero time) and the end of the incubation period.

To further characterize the reaction product (*p*-hydroxyphenylpyruvic acid) and also to check the validity of data obtained by use of the modified Briggs method, experiments were performed using L-[U-^{14}C]tyrosine. Prior to assay of the tyrosine aminotransferase activity of the 1-ml aliquots taken from the incubation system (at zero and 90 minutes of incubation at 37°), 0.5 μc of L-[U-^{14}C]tyrosine (New England Nuclear Corp.; 0.18 mc/mmole) was added to the assay-reaction flask in addition to unlabeled tyrosine. At the end of the 20-minute assay, *p*-hydroxyphenylpyruvic acid was estimated using 1 ml of the trichloroacetic acid supernatant, 1 ml of water, and 2 ml of Briggs "mixed reagent." The *p*-hydroxyphenylpyruvic acid present in the remaining trichloroacetic acid supernatant was further estimated by converting it to the 2,4-dinitrophenylhydrazone. The acid derivatives were prepared, extracted, and chromatographed on Whatman No. 1 paper according to the methods of Cavallini and Frontali (1954) and Strassman *et al.* (1960). After elution from the paper with dilute NH_4OH, the radioactivity of the 2,4-dinitrophenylpyruvic acid zone was measured using a proportional gas-flow counter (Nuclear Measurements Corp., Model PC-3A).

To determine whether the enzymatic activity was linear with increasing enzyme concentration, the rate of reaction as a function of volume of aliquot was investigated at zero time and after 90 minutes of incubation. In this case, the enzymatic activity of aliquots taken from the incubation system was measured continuously at 22° using the spectrophotometric method of Lin *et al.* (1958).

RESULTS

Several experiments were conducted using an incubation system containing the mitochondrial-microsomal-supernatant and a 30-minute incubation (37°) in air. In confirmation of a previous report (Diamondstone and Litwack, 1963a), addition of DPN$^+$ to the incubation system (1 μmole/ml) stimulated the appearance of tyrosine aminotransferase activity by about 50% over the controls containing no added pyridine nucleotide. Addition of either DPNH, TPN$^+$, or TPNH to the incubation system (1 μmole/ml) did not stimulate the appearance of enzymatic activity. Addition of either puromycin·2HCl, DL-ethionine, or RNAase to the incubation system at low or high concentrations did not inhibit the appearance of enzymatic activity.

Since increases in enzymatic activity consistently occurred during incubation under nitrogen, the presence of mitochondria was probably not essential to the appearance of aminotransferase activity, inasmuch as mitochondria function optimally in an highly aerobic environment. The average increase with the mitochondrial-microsomal-supernatant and a 30-minute incubation under nitrogen was 0.771 μmole of *p*-hydroxyphenylpyruvate formed per ml of mitochondrial-micro-

somal-supernatant, or 0.424 μmole of *p*-hydroxyphenylpyruvate formed per mg of Kjeldahl nitrogen. For the same system, except for incubation in an atmosphere of air, the average increase in enzymatic activity obtained from the three experiments was 0.831 μmole of *p*-hydroxyphenylpyruvate formed per ml of mitochondrial-microsomal-supernatant, or 0.434 μmole of *p*-hydroxyphenylpyruvate per mg of nitrogen. These results prompted further investigation of the appearance of enzymatic activity using the microsomal-supernatant under nitrogen.

Figure 1 shows that the appearance of tyrosine aminotransferease activity using the microsomal-supernatant is nearly linear. A maximum is attained at 90 minutes of incubation after which time the appearance falls off. The effects of puromycin, actinomycin D, or DPN^+ were tested in this system and the results are tabulated in Table I. The data show no significant inhibition in the appearance of enzymatic activity by puromycin and actinomycin D. On the other hand, DPN^+ enhanced the appearance of enzymatic activity.

Nuclear rat liver RNA was isolated according to Hoagland and Askonas (1963) 4 hours after injection of hydrocortisone (6 mg/100 g body wt), and also according to the procedure of Barondes *et al.* (1962) from untreated animals. When these preparations of RNA were added to the incubation system, appearance of tyrosine aminotransferase activity was not increased by more than 10% over the controls, and in some cases nuclear RNA inhibited the appearance of the enzymatic activity. Tests for nonenzymatic transamination were negative.

TABLE II

EFFECT OF *p*H OF INCUBATION AND OF 3′,5′-CYCLIC-AMP ON THE APPEARANCE OF TYROSINE AMINOTRANSFERASE ACTIVITY *in Vitro*[a]

Expt	*p*H of Incubation System	*p*-Hydroxyphenylpyruvate Formed per ml Microsomal-Supernatant per 90-Minute Incubation (μmoles) Control	Control + 0.03 mM	3′,5′-Cyclic-AMP
30	6.5	0.95		
37	6.5	0.20		
Average		0.57		
13	6.9	0.78	1.13	
16	6.9	0.61	0.93	
25	6.9	0.52	1.00	
27	6.9	0.87	1.20	
14	6.9	0.52	1.00	
Average		0.66	1.05	$P < 0.01$[b]
15	7.2	0.99		
30	7.2	0.49		
35	7.2	0.09		
Average		0.52		
27	7.5	0.52		
29	7.5	0.36		
Average		0.44		

[a] Incubation was carried out under nitrogen at 37° for 90 minutes at the indicated *p*H using the microsomal-supernatant. The value of enzymatic activity is derived from the 1.0-ml sample taken from the incubation system after 90 minutes and has been corrected by subtraction of the value of enzymatic activity derived from the 1.0-ml sample taken from the incubation system at zero time. In some experiments transferase activity was measured using the reduced-assay system (see Experimental). 3′,5′-Cyclic-AMP was added to the incubation system from fresh solution. The *p*H of the incubation system was checked before and after incubation. Slight variation in *p*H was noticed after the 90-minute incubation. Zero-time activity (activity without incubation) in these experiments was on the average 4.06 μmoles *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant or 5.48 μmoles of product formed per mg Kjeldahl nitrogen. [b] Statistical analysis was performed by comparing the values of the control with the values of the control plus 3′,5′-cyclic-AMP.

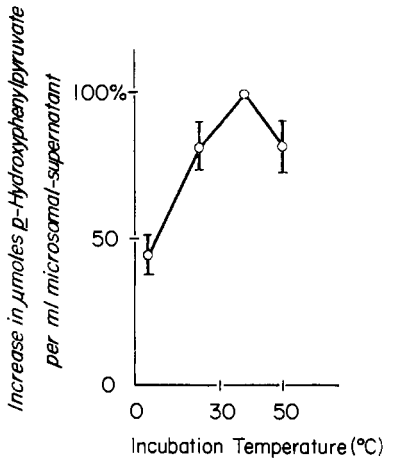

FIG. 2.—Effect of the temperature of incubation on the appearance of tyrosine aminotransferase activity. Incubation was carried out in air using a microsomal-supernatant. Tyrosine aminotransferase activity of the aliquots taken from the incubation system was measured using the reduced-assay system (see Experimental). The increases in μmoles of *p*-hydroxyphenylpyruvate formed during the 90-minute incubation over the zero-time controls (without incubation) are expressed as per cent of the maximal value obtained at 37°. Each point is the average of three to four experiments, except the point at 4° which is the average of two experiments. The vertical bars at each point represent the range of the increases in activity obtained with different experiments. The average increase in enzymatic activity over the zero-time controls for each temperature was 0.59, 1.05, 1.32, and 1.08 μmoles *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant at 4, 22, 37, and 50°, respectively. Aminotransferase activity at zero-time incubation (without incubation) was 4.23 μmoles *p*-hydroxyphenylpyruvate formed per ml microsomal-supernatant during a 20-minute enzymatic assay or 5.71 μmoles of product formed per mg Kjeldahl nitrogen.

The appearance of tyrosine aminotransferase activity using the microsomal-supernatant was tested as a function of temperature of incubation between 0 and 90 minutes. Figure 2 shows a dependence curve with an optimum at about 37°.

The appearance of enzymatic activity using a microsomal-supernatant was measured as a function of the *p*H of the incubation system between 0 and 90 minutes. 3′,5′-Cyclic-AMP at 0.03 mM was also added in the incubation system. The results of these experiments are recorded in Table II. A *p*H optimum at about *p*H 6.9 was observed. 3′,5′-Cyclic-AMP stimulated the appearance of enzymatic activity at the optimum *p*H to the extent of about 59%.

Table III shows data obtained using the microsomal-supernatant in which the aminotransferase activity was measured by the modified Briggs method, and also by the radioactivity of the dinitrophenylhydrazone of *p*-hydroxyphenylpyruvate formed in the enzymatic assay after 90 minutes of incubation. The radioactivity study confirms the results obtained with the Briggs reagent as reflected in the values of specific radioactivity for 0 and for 90 minutes of incubation.

Figure 3 shows that the rate of reaction is proportional to the concentration of the enzyme over the time of incubation employed. Tyrosine aminotransferase activity increases linearly with increasing enzyme

TABLE III

TYROSINE AMINOTRANSFERASE ACTIVITY AFTER INCUBATION FOR 0 AND 90 MINUTES (37°) AS MEASURED BY THE BRIGGS METHOD AND BY THE FORMATION OF DINITROPHENYLHYDRAZONE OF RADIOACTIVE *p*-HYDROXYPHENYLPYRUVIC ACID FORMED DURING A 20-MINUTE ENZYMATIC ASSAY[a]

	p-Hydroxyphenylpyruvate Formed per ml Incubation System (μmoles)[b]		Total cpm of 2,4-Dinitrophenylhydrazone of [U-^{14}C]-*p*-Hydroxyphenylpyruvic Acid Formed per ml of Incubation System				Specific Radioactivity (cpm/μmole *p*-hydroxyphenylpyruvate)	
	Time of Incubation		Time of Incubation					
			0 Min		90 Min		Time of Incubation	
Expt	0 Min	90 Min	Found	Expected[c]	Found	Expected[c]	0 Min	90 Min
1	1.44	2.17	25,700	26,600	40,200	40,100	29,800	30,900
2	1.75	2.64	23,500	32,600	36,400	48,800	22,300	23,000
3	2.00	4.35	36,000	37,000	68,800	82,500	30,000	26,500
4	1.44	3.34	21,000	26,600	52,500	60,000	24,400	26,300
Average	1.64[c]	3.12	26,500	30,700	49,500	57,800	26,600	26,700
		$P < 0.05$[d]			$P < 0.05$[d]			

[a] Incubation was carried out in air at 37° for 90 minutes using the microsomal-supernatant. Zero-time-activity values (activity without incubation) have not been subtracted from the activity values obtained after the 90-minute incubation. Transferase activity of the aliquots taken from the incubation system was measured using the reduced-assay system (see Experimental). Dinitrophenylhydrazone of the formed [U-^{14}C]-*p*-hydroxyphenylpyruvate was prepared, extracted, and measured as described in the text. [b] Measured by the Briggs modified method. [c] Calculated on the basis of μmoles of *p*-hydroxyphenylpyruvic acid estimated by the Briggs method. Animals used in the study were obtained from Carworth Farms, whereas animals used in previous studies were obtained from Huntingdon Farms. This fact, together with differences in the air-conditioning, may account for the smaller zero-time values reported in this table. [d] Tests of significance were performed as indicated in Table II. The activity values obtained at zero-time and 90-minute incubation were compared. The same treatment was applied to radioactivity values.

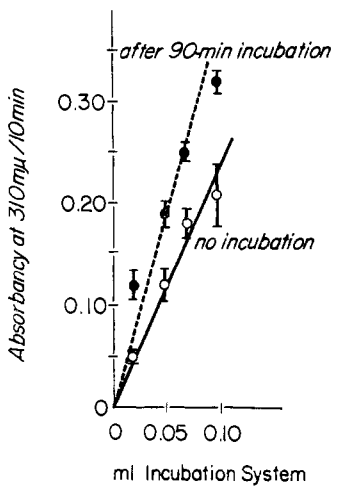

FIG. 3.—The velocity of the reaction is plotted against enzyme concentration. Each point is the average of 4 experiments. The vertical bars at each point represent the standard error of the mean $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$. Enzyme assays were performed spectrophotometrically according to Lin *et al.* (1958) at 22°. Triplicate assays with constant enzyme concentration from the same preparation gave essentially identical time-course curves. Assays were performed using the microsomal-supernatant at zero time (without incubation) and after 90 minutes of incubation at 37° in air.

concentration at zero time and also after 90 minutes of incubation.

DISCUSSION

The appearance of tyrosine aminotransferase activity occurring during incubation of cell-free systems from rat liver could result in several ways, such as: (a) release of a bound form of the enzyme from the microsomal or soluble fractions, or from both; (b) an incubation-dependent activation of the apoenzyme; (c) an incubation-dependent removal of an inhibitor; or (d) a physical change of the protein to a more active state. An apparent inactivation of the incubation-dependent appearance of the tyrosine aminotransferase activity appears after the maximum has been reached, shown by Figure 1. An abrupt decline is observed after 90 minutes of incubation, which may reflect inactivation of the enzyme. An incubation-dependent activation of tryptophan pyrrolase activity, which required ATP, was reported by Pitot and Cho (1961). ATP was always present in the incubation system used here, but ATP was not demonstrated to be essential for the appearance of transferase activity.

In confirmation of a previous report (Diamondstone and Litwack, 1963a), appearance of tyrosine aminotransferase activity is stimulated by DPN$^+$ when it is present in cell-free systems during incubation (Table I). Stimulation by DPN$^+$ (about 50% or more) occurs whether the mitochondria are present or absent and when an atmosphere of nitrogen is used. The stimulation by DPN$^+$ is specific insofar as DPNH, TPN$^+$, and TPNH have no stimulatory effect.

Puromycin and actinomycin D do not significantly inhibit the appearance of enzymatic activity. The observed small inhibition by puromycin suggests that *de novo* synthesis of new enzyme protein does not occur during incubation, but rather that an indirect effect occurs, since at the concentrations tested this compound is a potent inhibitor of protein synthesis. The appearance of transferase activity during incubation is stimulated by 3′,5′-cyclic-AMP. In the presence of ATP, the activity of partially purified phosphofructokinase from guinea pig heart and also phosphofructokinase activity of homogenates from different sources are stimulated by the cyclic nucleotide (Mansour, 1963; and Mansour and Mansour, 1962). 3′,5′-Cyclic-AMP was also shown to reactivate tryptophan pyrrolase activity after heating (Chytil and Skrivanova, 1963) and to be involved in the regulation of liver phosphorylase (Rall and Sutherland, 1961). In addition, Mansour (1964) recently reported that guinea pig heart phosphofructokinase exists in two forms, and that 3′,5′-cyclic-AMP enhances the activity of the acidified enzyme considerably. In light of these reports, it may be that the cyclic nucleotide is acting upon tyrosine aminotransferase in a way similar to its action on other

enzymes; however, direct evidence on this point is not yet available.

The uncompetitive inhibition exhibited by thyroxine upon tyrosine aminotransferase activity at high pyridoxal phosphate concentrations in contrast to the stimulatory effect of thyroxine on the enzyme at low coenzyme concentrations (Diamondstone and Litwack, 1963b) and the similar inhibitory effect of thyroxine on glutamic dehydrogenase which results in dissociation of this enzyme into two congeners (Wolff, 1962) may also suggest that tyrosine aminotransferase exists in various degrees of polymerization. In view of the involvement of 3′,5′-cyclic-AMP on the regulation of liver phosphorylase (Rall and Sutherland, 1961), pig heart phosphofructokinase (Mansour, 1964), and liver tyrosine aminotransferase (this report), the action of the cyclic nucleotide may be of a regulatory nature in connection with enzymatic dissociation and association. However, the degree of stimulation by 3′,5′-cyclic-AMP of tyrosine aminotransferase activity is not at all comparable to that of phosphofructokinase (Mansour and Mansour, 1962), but it is similar in degree to the stimulation of tryptophan pyrrolase activity (Chytil and Skrivanova, 1963).

The appearance of tyrosine aminotransferase activity observed during incubation has been determined by the measurement of radioactivity of the dinitrophenylhydrazone of the reaction product, *p*-hydroxyphenylpyruvate. Increases in radioactivity correspond to increases of product measured by colorimetry. These measurements substantiate the appearance of enzymatic activity upon incubation.

Statistical analyses (Bernstein and Weatherall, 1952) were performed by comparing the increases in aminotransferase activity obtained at zero time and after 90 minutes of incubation. The results showed a highly significant difference ($P < 0.01$) between control values and values obtained with added 3′,5′-cyclic-AMP. Similar results ($P < 0.05$) were obtained in the case o-radioactivity data owing to significant differences between enzymatic-activity values at 90 minutes' incubation and values obtained without incubation. Alf though statistical analysis of control values and values obtained with added DPN^+ did not show great significance ($P < 0.2$), addition of DPN^+ to the incubation system usually resulted in higher enzymatic activity.

Nonspecific stimulation of tyrosine aminotransferase activity *in vivo* by various biological agents, including RNA, has been shown to depend on the presence of the adrenal gland or of hydrocortisone administration (Litwack and Diamondstone, 1962). That hydrocortisone may directly affect the induction system was recently inferred by the studies of Litwack *et al.* (1963), who reported that the subcellular distribution of enzymatic activity (4 hours after hydrocortisone administration) is similar to the subcellular distribution of hydrocortisone at its earlier peak concentration. Goldstein *et al.* (1962) showed that puromycin completely inhibited the rise of tyrosine aminotransferase activity due to hydrocortisone administration in a perfused-liver system and that this inhibition was unaffected by an inducing level of hydrocortisone. This finding suggests that hydrocortisone may act directly on the liver cell. Garren and Howell (1963) also reported that puromycin inhibits tyrosine aminotransferase activity formation *in vivo*. The available evidence indicates that the system developing tyrosine aminotransferase activity *in vitro* is no longer representative of the *in vivo* process since it contains only a small or negligible component sensitive to the inhibitors of protein synthesis, it is not responsive to addition of informational RNA, and it has become insensitive to or inhibited by hydrocortisone, at the level tested. In spite of the differences between the *in vitro* system described here and observations which have been made *in vivo*, use of the cell-free system may lead to a clearer understanding of some of the factors involved in the regulation of enzymatic activity at the molecular level.

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