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# The Regulation of Phenylalanine Hydroxylase in Rat Tissues *in vivo*

## SUBSTRATE- AND CORTISOL-INDUCED ELEVATIONS IN PHENYLALANINE HYDROXYLASE ACTIVITY

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Injections of phenylalanine increased by 2.5-fold in 9h the hepatic phenylalanine hydroxylase activity of 6-day-old or adult rats that had been pretreated (24h earlier) with *p*-chlorophenylalanine; without such pretreatment, phenylalanine did not raise the enzyme concentration. This difference is paralleled by the much greater extent to which the injected phenylalanine accumulated in livers of the pretreated compared with the normal animals. The hormonal induction of hepatic phenylalanine hydroxylase activity obeyed different rules: an injection of cortisol was without effect on adult livers but caused a threefold rise in phenylalanine hydroxylase activity of immature ones, both without and after pretreatment with *p*-chlorophenylalanine. In the latter instance, the effects of cortisol, and of phenylalanine were additive. Actinomycin inhibited the cortisol- but not the substrate-induced increase of phenylalanine hydroxylase, whereas puromycin inhibited both. The results indicate that substrate and hormone, two potential positive regulators of the amount of the hepatic (but not the renal) phenylalanine hydroxylase, act independently by two different mechanisms. The negative effector, *p*-chlorophenylalanine, also appears to interact with the synthetic (or degradative) machinery rather than with the existing phenylalanine hydroxylase molecules: 24h were required *in vivo* for an 85% decrease to ensue, and no inhibition occurred *in vitro* when incubating the enzyme with *p*-chlorophenylalanine or with liver extracts from *p*-chlorophenylalanine-treated rats.

Among the several studies on the regulation of phenylalanine hydroxylase in rat liver *in vivo*, there is one (Freedland *et al.*, 1962) that suggests a positive relationship between the amount of ingested phenylalanine and the concentration of this enzyme; they reported that in rats maintained on a low phenylalanine diet for 7 days the hepatic phenylalanine hydroxylase activity was decreased by 43%. However, the ingestion of large amounts of phenylalanine did not appear to exert the opposite effect: a single administration of phenylalanine (15mg/10g) did not raise the hepatic phenylalanine hydroxylase activity (Freedland *et al.*, 1962), and 4 weeks of a high (7%) phenylalanine diet caused a 40% decrease in activity (Freedland *et al.*, 1964). The present re-investigation of the possible regulatory role of the substrate was prompted by an accidental observation; in experiments designed to maintain high concentrations of phenylalanine in the plasma of immature rats [see the preceding paper (DelValle & Greengard, 1976)] we noted that the recovery of phenylalanine hydroxylase after inhibition by *p*-chlorophenylalanine *in vivo* was greatly hastened by injections of phenylalanine. Evidence is now presented for a substrate-mediated regulation of the amount of enzyme. Experiments with *p*-chlorophenylalanine relevant to the mechanism by which it decreases the phenylalanine hydroxylase activity are also included here, whereas those which served the practical purpose of creating hyperphenylalaninaemic rats are described in the preceding paper (DelValle & Greengard, 1976).

McGee *et al.* (1972*a*) showed that an injection of cortisol to suckling rats increases the activity of hepatic phenylalanine hydroxylase. In view of the new observations on the regulation of the enzyme, it was decided to compare the mode of action of cortisol and phenylalanine, and to test the hormone effect in rats treated with the inhibitory analogue, *p*-chlorophenylalanine. The results demonstrate distinct differences between the mechanism of the substrate- and the hormone-induced elevations of hepatic phenylalanine hydroxylase.

### Materials and Methods

The animals used and the preparation of the phenylalanine and *p*-chlorophenylalanine suspension to be injected were as described in the preceding paper (DelValle & Greengard, 1976). Cortisol (hydrocortisone acetate; Merck, Sharp and Dohme, Rahway, NJ, U.S.A.), puromycin (dihydrochloride, Nutritional Biochemicals Corporation, Cleveland,

OH, U.S.A.) and actinomycin D (Merck, Sharp and Dohme, West Point, PA, U.S.A.) were suspended in 0.9% NaCl. All injections were given intraperitoneally.

Freshly excised tissues were homogenized in 9 vol. of 0.15 M-KCl, and centrifuged at 16000*g* for 15 min. The supernatant fraction was assayed for phenylalanine hydroxylase activity as described by McGee *et al.* (1972*a*). Reaction mixtures of final volume 1 ml contained 0.025–0.2 ml of tissue extract, phenylalanine (10 mM), 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (0.75 mM), potassium phosphate buffer, pH 6.8 (0.1 M) and dithiothreitol (5 mM). The tyrosine formed was determined by the method of Udenfriend & Cooper (1952) as modified by McGee *et al.* (1972*a*). The phenylalanine hydroxylase activities are expressed in units (nmol of tyrosine formed/min at 25°C).

To determine the hepatic phenylalanine and tyrosine concentrations, freshly excised livers were homogenized in 4 vol. of trichloroacetic acid (final concentration 0.3 M). The precipitated protein was left for 20 min at room temperature, then sedimented in a clinical centrifuge, and the supernatant fluid was assayed in an automatic amino acid analyser as described in the preceding paper (DelValle & Greengard, 1976).

## Results

Since several experiments on the induction of hepatic phenylalanine hydroxylase to be described used rats that had been pretreated with *p*-chlorophenylalanine, some observations relating to the mode of action of this substrate analogue itself will be described first. Table 1 confirms that *p*-chlorophenylalanine is not an effective inhibitor of phenylalanine hydroxylase activity *in vitro*: liver extracts from 6-day-old rats [as those of adult rats (Guroff, 1969)] exhibited little inhibition in the presence of 8 mM-*p*-chlorophenylalanine; 0.8 mM-*p*-chlorophenylalanine was without any effect even if preincubated

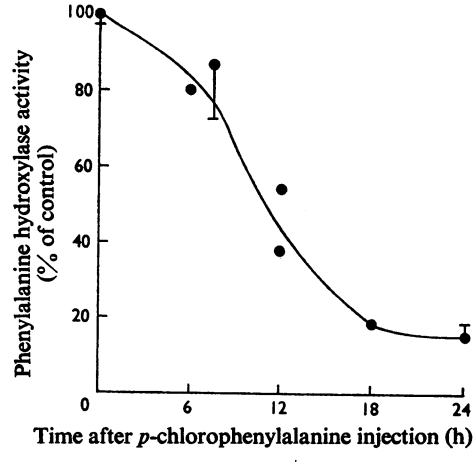


Fig. 1. *Time-course of inhibition of hepatic phenylalanine hydroxylase by p-chlorophenylalanine in vivo*

Rats (6 days old) were injected with 18 μmol of *p*-chlorophenylalanine/10 g body wt. The phenylalanine hydroxylase activities (units/g of liver), assayed at the indicated times thereafter, are given as a percentage of that found in the untreated controls (i.e. 562 units/g). The points refer to individual animals or to means of results with three or four animals, the vertical bar representing 1 S.D.

Table 1. *Attempts to inhibit phenylalanine hydroxylase activity in vitro*

Liver supernatants were from 6-day-old rats, untreated (normal) or 24 h after the administration of 18 μmol of *p*-chlorophenylalanine per 10 g (*p*-chlorophenylalanine-treated); where indicated, a portion of the latter was deproteinized by boiling. The usual assay system contained 0.1 ml of one, or 0.1 ml of each, of the indicated preparations. '+ *p*-Chlorophenylalanine' refers to the addition of *p*-chlorophenylalanine (final concns. shown) to the usual assay system at the beginning of the incubation. Experiments of the last two lines of the table involved preincubation (45 min at 25°C) of whole homogenates in the absence of phenylalanine, without or with 25 mM-*p*-chlorophenylalanine, followed by centrifugation at 100000*g* for 50 min and assay of the supernatants in the standard manner.

| Liver extracts | Modifications of assay system | Hepatic phenylalanine hydroxylase activity (units/assay) |
|---|---|---|
| Normal | — | 4.9 |
| *p*-Chlorophenylalanine-treated | — | 1.7 |
| Normal + *p*-chlorophenylalanine-treated | — | 6.9 |
| Normal + *p*-chlorophenylalanine-treated, deproteinized | — | 4.9 |
| Normal | +8 mM-*p*-Chlorophenylalanine | 4.1 |
| Normal | +0.8 mM-*p*-Chlorophenylalanine | 5.0 |
| Normal | Preincubated without *p*-chlorophenylalanine | 4.9 |
| Normal | Preincubated with *p*-chlorophenylalanine | 4.7 |

with liver homogenates in the absence of phenylalanine (see the last two lines, Table 1). The concentration of *p*-chlorophenylalanine in liver, determined 24h after its administration, was only 0.5 $\mu$mol/g. Thus the consequent *p*-chlorophenylalanine concentration (0.01 mM) in assay mixtures containing samples of such livers is not responsible for their low phenylalanine hydroxylase activity (see Fig. 1); nor is there any sign of the formation of inhibitory metabolites *in vivo*, since admixtures with normal liver extracts exhibited the expected sum of the individual activities (Table 1).

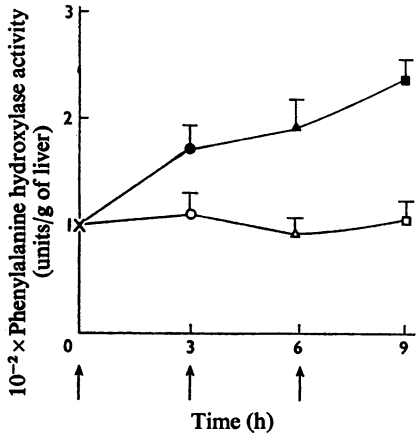


Fig. 2. *Substrate-induced rise of phenylalanine hydroxylase activity in p-chlorophenylalanine-treated rats*

Rats (6 days old) received 9 $\mu$mol of *p*-chlorophenylalanine/10g body wt. A day later (0h on the abcissa) some were killed (×). Others were injected with 13 $\mu$mol of phenylalanine/10g (●, ▲, ■), or with 0.9% NaCl (○, △, □), once (●, ○), twice (▲, △), or three times (■, □) at 3h intervals (indicated by the arrows) and were killed 3h after the last injection.

Previous studies (Guroff, 1969) with *p*-chlorophenylalanine *in vivo* centred mainly on the rate of the eventual recovery of the hepatic phenylalanine hydroxylase activity and did not provide information on the initial rate of loss of the enzyme. Fig. 1 shows that the loss of activity is a slow process; 7h after the administration of *p*-chlorophenylalanine there was only a 20% inhibition; more than 18h were required to attain minimum activity.

In all subsequent experiments, as in those of Fig. 1, *p*-chlorophenylalanine was injected in amounts (9–18 $\mu$mol/10g body wt.) necessary to cause a maximum decrease in hepatic phenylalanine hydroxylase activity in 24h [see the preceding paper (DelValle & Greengard, 1976)]. The activity remained suppressed for another day (DelValle & Greengard, 1976). However, injections of phenylalanine during this time caused a rapid upturn; Fig. 2 shows that in 9h the activity rose from 104 to 235 units/g. This substrate-induced elevation was inhibited by the administration of puromycin but not by actinomycin (Table 2). Fig. 2 also shows that induction occurred in adult as well as in 6-day-old rat livers but not in immature rat kidneys (adult rat kidneys were not tested).

The last part of Table 2 shows that in normal rats, in contrast with rats pretreated with *p*-chlorophenylalanine, injections of phenylalanine did not raise the concentration of phenylalanine hydroxylase. This difference is paralleled by the different ability of the two types of livers to concentrate phenylalanine. Normal rats, owing to their high phenylalanine hydroxylase activity, eliminate injected phenylalanine rapidly; at 2.5h, the concentration of phenylalanine in liver, as in plasma (McGee *et al.*, 1972*b*), was no longer elevated. Even at 30–60min the increase was much less than that seen in *p*-chlorophenylalanine-

Table 2. *Effect of phenylalanine injections on phenylalanine hydroxylase activity*

Pretreatment consisted of one injection of 9 $\mu$mol of *p*-chlorophenylalanine/10g body wt. 24h before the beginning of injections of 0.9% NaCl or 13 $\mu$mol of phenylalanine/10g body wt. Three such injections were given at 3h intervals, assays were done 3h after the last. When indicated, puromycin (5–7.5mg/100g) and actinomycin (0.1mg/100g) were given twice, 1h before and 3h after the first injection of phenylalanine. Values are means ±S.D. with the numbers of animals in parentheses.

| | | Phenylalanine hydroxylase activity (units/g) | | | |
|---|---|---|---|---|---|
| | | Liver | | Kidney | |
| Pretreatment | Injections | 6-day-old | Adult | 6-day-old | Adult |
| *p*-Chlorophenylalanine | NaCl | 104±23 (9) | 96±18 (3) | 68±15 (6) | 98± 8 (3) |
| *p*-Chlorophenylalanine | Phenylalanine | 235±19 (9) | 215± 24 (3) | 50±13 (3) | — |
| *p*-Chlorophenylalanine | Phenylalanine+puromycin | 152±29 (10) | — | — | — |
| *p*-Chlorophenylalanine | Phenylalanine+actinomycin | 218±11 (3) | 190±19 (3) | — | — |
| None | NaCl | 620±52 (7) | 946±98 (5) | 115±18 (6) | 156±16 (3) |
| None | Phenylalanine | 592, 517 | 958±24 (3) | 98± 2 (4) | 201±10 (3) |

Table 3. *Hepatic phenylalanine and tyrosine concentrations after injections of p-chlorophenylalanine and/or phenylalanine*

Pretreatment of 6-day-old rats was as described in Table 2; 24h later, rats from each group were injected with 13 $\mu$mol of phenylalanine/10g body wt. and were killed at the indicated time thereafter. Others were given 0.9% NaCl at the same time (None) and killed 2.5h later. Livers were homogenized immediately and analysed as described under 'Methods'. Values are means ±S.D. of results with three rats.

| Pretreatment | Time of assay after phenylalanine injection (h) | Concentration (nmol/g of liver) | |
|---|---|---|---|
| | | Phenylalanine | Tyrosine |
| None | None | 138± 5 | 200± 31 |
| None | 0.5 | 591± 25 | 1430±200 |
| None | 1.0 | 348±107 | 723± 64 |
| None | 2.5 | 127± 16 | 330± 58 |
| *p*-Chlorophenylalanine | None | 197± 21 | 245± 22 |
| *p*-Chlorophenylalanine | 2.5 | 720± 95 | 397±100 |

Table 4. *The induction of phenylalanine hydroxylase by cortisol with and without p-chlorophenylalanine, phenylalanine and antibiotics*

Injections (beginning 1 day after pretreatment, as in Table 2) consisted of a single dose of 0.25mg of cortisol/10g body wt. or of three injections of phenylalanine, or both. The times between the first of these injections and assay are given in parentheses. The amounts of phenylalanine, puromycin and actinomycin D were as in Table 2. The last two substances were injected twice, 1h before and 3h after cortisol. Values refer to individual animals or to means ±S.D., with the numbers of animals in parentheses.

| Pretreatment | Injections | Phenylalanine hydroxylase activity (units/g of liver) | |
|---|---|---|---|
| | | 6-day-old | Adult |
| None | None | 680±52 (7) | 1013±79 (3) |
| None | Cortisol (24h) | 1581±84 (4) | 978±76 (3) |
| None | Cortisol+actinomycin (24h) | 788±41 (3) | — |
| None | Cortisol (9h) | 1026±71 (3) | — |
| None | Cortisol+puromycin (9h) | 682±31 (3) | — |
| None | Cortisol+actinomycin (9h) | 662±51 (3) | — |
| None | Cortisol+phenylalanine (9h) | 867±68 (3) | — |
| *p*-Chlorophenylalanine | None | 103±21 (10) | 80±18 (4) |
| *p*-Chlorophenylalanine | Cortisol (24h) | 248, 275 | 96± 9 (4) |
| *p*-Chlorophenylalanine | Cortisol (9h) | 188±19 (3) | — |
| *p*-Chlorophenylalanine | Phenylalanine (9h) | 235±19 (9) | — |
| *p*-Chlorophenylalanine | Cortisol+phenylalanine (9h) | 347±15 (4) | — |

treated rats 2.5h after an injection of phenylalanine (Table 3). Thus during the 9h-induction experiments (Fig. 2 and Table 2) there were only small, transient rises in the phenylalanine concentrations of normal livers, whereas in *p*-chlorophenylalanine-treated rats, high concentrations of hepatic phenylalanine prevailed for 2.5h after each injection, i.e. throughout most of the 9h period of rise of the phenylalanine hydroxylase activity. Additional experiments with *p*-chlorophenylalanine-treated rats showed that injections of phenylalanine given at intervals longer than 3h did not induce the enzyme, again indicating a close association between the persistence of increased hepatic phenylalanine and the augmentation of the enzyme concentration.

The last column of Table 3 indicates that, as expected, phenylalanine administration increased the amount of tyrosine in the liver. Much more tyrosine must have been produced in the normal than in the *p*-chlorophenylalanine-treated rats but this difference is no longer present by 2.5h. At this time, in *p*-chlorophenylalanine-treated rats, the phenylalanine concentration was still in the process of decline and this is probably the reason for the individual variations (see the S.D. values in the last line of Table 3) of both the phenylalanine and the tyrosine values; the sums of the two amino acid concentrations in these three livers were almost identical (1120, 1100 and 1130 nmol/g).

The experiments of Table 4 compare the hormonal induction of hepatic phenylalanine hydroxylase with its substrate induction (Table 2). An injection of cortisol was known to double in 24h the enzyme concentration in 13-day-old rats (McGee *et al.*,

1972*a*). In 6-day-old rats, too, the activity rose from 680 to 1581 in 24h and to 1026 units/g in 9h. Puromycin or actinomycin almost completely prevented the cortisol-induced increase in phenylalanine hydroxylase activity (Table 4). Injections of phenylalanine (which in these normal rats did not cause sustained elevations of hepatic phenylalanine and hydroxylase activity) did not enhance the effect of cortisol.

Table 4 also demonstrates that the percentage increase in phenylalanine hydroxylase activity caused by cortisol was similar in normal and in *p*-chlorophenylalanine-treated, immature rats; in the latter the basal activity was only 103 units/g and it rose to 250 units/g in 24h. The last three lines of Table 4 demonstrate that in *p*-chlorophenylalanine-treated rats the effects of cortisol and the substrate were additive: combined treatment caused an increase of 244 units/g (over the basal 103 units/g) as compared with the increments of 85 units with cortisol and 132 units with phenylalanine.

A further indication of the independent and different actions of cortisol and phenylalanine is that the former was ineffective in adult rats (either normal or *p*-chlorophenylalanine-treated; Table 4, last column) whereas phenylalanine caused similar increases of hepatic phenylalanine hydroxylase in adult and in immature *p*-chlorophenylalanine-treated rats (see Table 2).

## Discussion

The administration of *p*-chlorophenylalanine was thought to cause an irreversible inhibition of the hepatic phenylalanine hydroxylase activity since the eventual, spontaneous return of the activity necessitated protein synthesis *de novo* (Guroff, 1969). However, it has not been possible to explain the mechanism of the inhibition itself because *p*-chlorophenylalanine was without effect on the enzyme *in vitro* (Guroff, 1969) even, as shown here, when preincubated with liver homogenates in the absence of phenylalanine. It is unlikely that the inhibition *in vivo* depends on non-hepatic functions, since *p*-chlorophenylalanine also decreased the phenylalanine hydroxylase activity of hepatoma cells when present for several hours in the culture medium (Miller *et al.*, 1975). Nor can the phenomenon be attributed to the gradual accumulation of dissociable, inhibitory metabolites in the liver (or the elimination of unknown activators) because admixtures of extracts from normal and *p*-chlorophenylalanine-treated rats exhibited the expected sum of phenylalanine hydroxylase activity (Table 1). Another important new observation is that the loss, like the restoration (Guroff, 1969), of phenylalanine hydroxylase activity *in vivo* is a very slow process; an 85% decrease required 24h. We therefore believe that the administered *p*-chlorophenylalanine (and/or its metabolites) does not interact with existing phenylalanine hydroxylase molecules but may interfere with their synthesis. Gál & Millard (1973) suggested that *p*-chlorophenylalanine replaces phenylalanine in the active region of the nascent enzyme, resulting in decreased catalytic potential. There is no evidence, however, that *p*-chlorophenylalanine is incorporated into the phenylalanine hydroxylase or that the active region of the molecule contains phenylalanine. Nevertheless, the question of whether the decreased activity is due to a smaller number of enzyme molecules or to a less efficient enzyme species has to be left open. In either case, we categorize *p*-chlorophenylalanine as a 'synthetic regulator' in that its influence (be it on rates of synthesis or degradation, or quality of the nascent enzyme) is restricted to the dynamic state of turnover in the living cell.

The natural substrate, phenylalanine, and cortisol are also synthetic regulators of phenylalanine hydroxylase, albeit positive ones. The rise brought about with either agent is a time-consuming process though much more rapid than the spontaneous restoration of the enzyme concentration after *p*-chlorophenylalanine treatment (Guroff, 1969), and can be inhibited by puromycin. We refer to this type of phenomenon as 'induction' (Greengard, 1967) with the understanding that it may reflect either enhanced synthesis and/or decreased rate of degradation of the enzyme (or perhaps the formation of a more active molecular species).

Although phenylalanine was as effective in adult as in immature (*p*-chlorophenylalanine-treated) rats, the cortisol induction was restricted to the latter. Decreases or increases with age in responsiveness to a hormone (Greengard, 1971) are by no means unique to phenylalanine hydroxylase. Arginase (Greengard *et al.*, 1970) and ornithine aminotransferase (Herzfeld & Greengard, 1969), for example, can be induced by a single injection of cortisol in suckling but not in adult rats. Inhibition by actinomycin (Table 4; presumably reflecting inhibition of RNA synthesis) is also a common feature of hormone-induced increases of enzyme amounts *in vivo* (Greengard, 1967). Nor is it unusual for the agent to raise the amount of an enzyme in one tissue of the organism and not in other tissues containing comparable basal concentrations of the same enzyme (Greengard, 1971). The renal phenylalanine hydroxylase did not respond to cortisol (nor to phenylalanine) injections although it appears to have the same catalytic and immunological properties as the hepatic enzyme (Ayling *et al.*, 1974) and is also inhibited, though to a lesser extent, by *p*-chlorophenylalanine (Table 2).

The dependence on age and on uninhibited RNA synthesis are two different features that clearly distinguish the cortisol from the substrate induction of phenylalanine hydroxylase. The additive effect of

hormone and substrate (in immature, *p*-chlorophenylalanine-treated rats) further indicates that they act by two different mechanisms. A striking contrast is that cortisol caused the same percentage increase of phenylalanine hydroxylase activity in normal and in *p*-chlorophenylalanine-treated suckling rats, whereas phenylalanine was ineffective in the former. Thus *p*-chlorophenylalanine does not seem to interfere with the site at which cortisol acts; whether this analogue and the natural substrate (appearing to exert the opposite effects) share a common site of impact remains to be investigated.

The dual regulation of hepatic phenylalanine hydroxylase by cortisol and substrate is reminiscent of that of tryptophan oxygenase. Cortisol and tryptophan induce this rat liver enzyme by two different mechanisms (Civen & Knox, 1959; Greengard & Feigelson, 1961; Schimke *et al.*, 1965); their effects are additive (Knox & Piras, 1967) and actinomycin inhibits the hormone-induced rise only (Greengard *et al.*, 1963). However, demonstration of the substrate induction of tryptophan oxygenase did not require any pretreatment that lowers its basal value. A possible explanation for the need of such pretreatment in the case of phenylalanine hydroxylase may be arrived at by considering the tissue concentration of the inducer as an important variable. The tryptophan oxygenase remains elevated for several days after the administration of the non-metabolizable α-methyltryptophan (Greengard, 1964); the rise in the amount of enzyme in response to an injection of the natural substrate is restricted to the period during which the intrahepatic substrate concentration is high, 300 and 20 times above normal at 1 and 3h respectively (Civen & Knox, 1959). An elevation (though of lower magnitude) of hepatic phenylalanine persisted in our *p*-chlorophenylalanine-treated rats for 2.5h after an injection of phenylalanine; even so, at least one more injection (3h after the first one) was required to cause an appreciable rise in phenylalanine hydroxylase activity. Without *p*-chlorophenylalanine pretreatment the hepatic phenylalanine concentration was back to normal in 2.5h and even at 1h it was less than three times above normal. One would hardly expect that such small evanescent rises of substrate concentration could increase the amount of phenylalanine hydroxylase, which has a much longer half-life (cf. Guroff, 1969; Schimke *et al.*, 1965) and a much more sluggish response to cortisol (Table 4) than does tryptophan oxygenase (Civen & Knox, 1959).

The above considerations have an important implication for the validity of the numerous past experiments, carried out by ourselves and other workers, in which the injection of a rapidly catabolizable substrate did not cause enzyme induction. Such negative observations merely indicate that a temporary increase in substrate metabolism was without effect. Whether or not an increased concentration of substrate would induce (and this is relevant to basic mechanisms) can only be tested in a situation where its intracellular content has been increased to an appreciable extent and for a period commensurate with the potential rate of synthesis, relative to degradation, of the enzyme.

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