DEVELOPMENTAL ASPECTS OF TYROSINE METABOLISM

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DEDICATION

To my wife Hattie and son Maghimbi for their patience, understanding and continual encouragement.

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ABSTRACT

The developmental patterns of the enzymes involved in the metabolic degradation of tyrosine in rat liver, tyrosine transaminase, p-hydroxyphenylpyruvate hydroxylase, homogentisate oxygenase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase were studied during the first 24 hours after birth. Except for tyrosine transaminase, all were quite active at birth. The developmental changes indicated that the transaminase, isomerase, and hydrolase did not appear to show coordinated patterns in this time-period. However, the hydroxylase and oxygenase appeared to be coordinated in their development, an observation which is consistent with the results of the analysis of variance. With the exception of the isomerase, each enzyme showed a peak of activity at some point in the 24 hours; these peaks appeared to be timed in the reverse of the metabolic sequence. The first enzyme of the metabolic sequence (transaminase) had the lowest activity at most times, the last enzyme (hydrolase) had

the highest activity, with the remaining three enzymes falling in between these extremes. No satisfactory explanation is available for the very high activity of the hydrolase.

INTRODUCTION

The route for oxidative degradation of tyrosine to acetoacetate and fumarate is quantitatively the most important pathway, accounting for the bulk of tyrosine catabolized daily under normal physiological conditions. This route is of particular interest in that a number of diseases resulting from "inborn errors of metabolism" are associated with it. This pathway also accounts for the classification of tyrosine as both ketogenic and glucogenic.

The metabolic sequence to be discussed is catalyzed by five enzymes. Their official and unofficial names are given in Table 1.

The overall metabolic sequence of tyrosine is shown in Figure 1. This amino acid is converted reversibly into p-hydroxyphenylpyruvate by a process of transamination with α-ketoglutarate, catalyzed by tyrosine transaminase. The transaminase is inducible by glucocorticoid hormones, its coenzyme-precursor, pyridoxine, and under certain conditions, by its substrate (discussed in detail in the next

TABLE 1

LIST OF ENZYMES (1)

EC NUMBER	SYSTEMATIC NAME	TRIVIAL NAME	NAME USED IN THESIS
2.6.1.5	L-Tyrosine: 2-oxoglutarate aminotransferase	Tyrosine aminotransferase	Tyrosine transaminase
1.14.2.2	p-Hydroxyphenylpyruvate, ascorbate: oxygen oxidoreductase (hydroxylating)	p-Hydroxyphenylpyruvate hydroxylase	p-Hydroxyphenylpyruvate hydroxylase
1.13.1.5	Homogentisate: oxygen oxidoreductase	Homogentisate oxygenase	Homogentisate oxygenase
5.2.1.2	4-Maleylacetoacetate *cis-trans*-isomerase	Maleylacetoacetate isomerase	Maleylacetoacetate isomerase
3.7.1.2	4-Fumarylacetoacetate fumarylhydrolase	Fumarylacetoacetase	Fumarylacetoacetate hydrolase

Figure 1

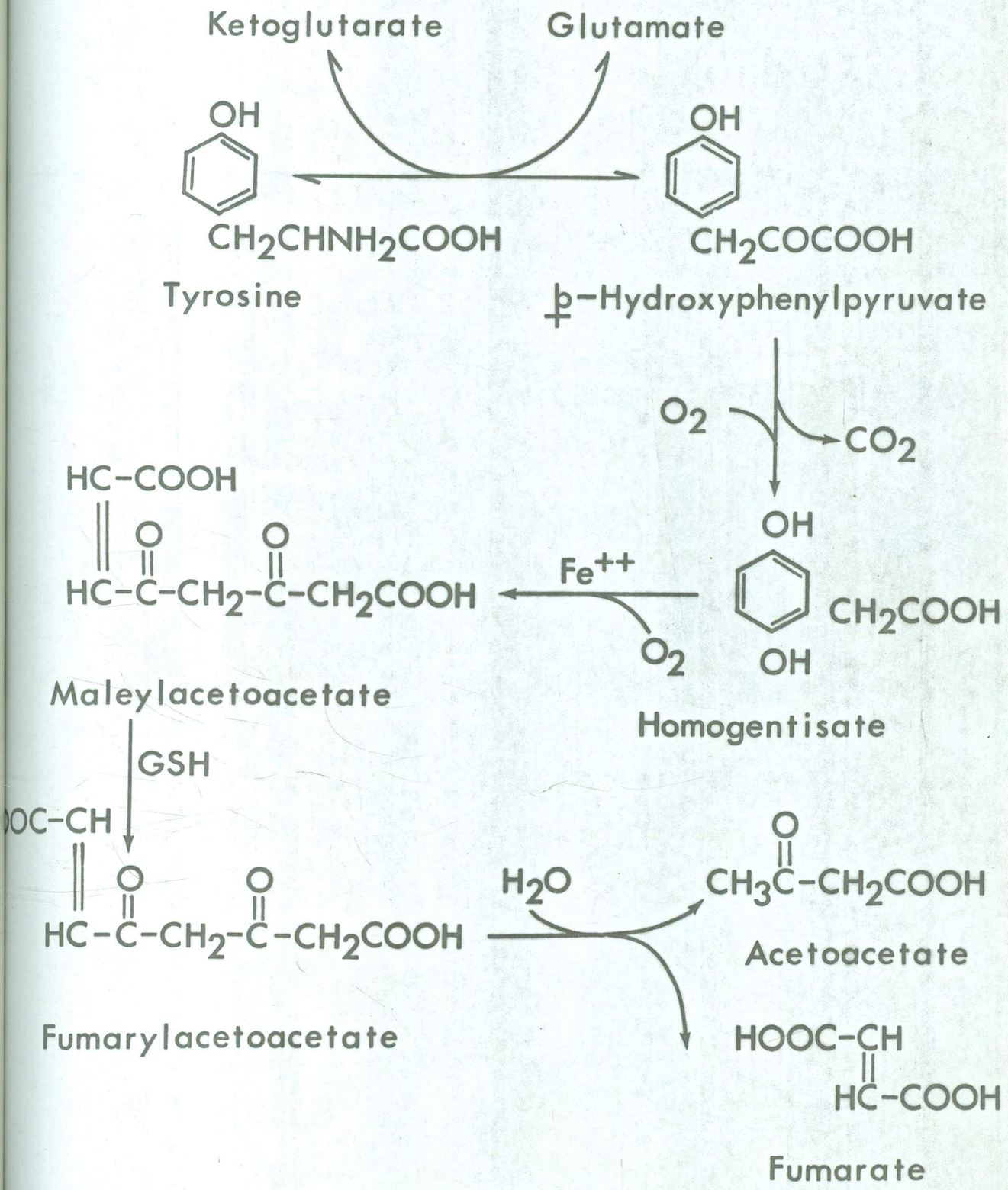

Ketoglutarate
Glutamate
OH
OH
CH2CHNH2COOH
CH2COCOOH
Tyrosine
p-Hydroxyphenylpyruvate
O2
CO2
OH
HC-COOH
HC-C-CH2-C-CH2COOH
Fe++
CH2COOH
O2
OH
Maleylacetoacetate
Homogentisate
GSH
OC-CH
HC-C-CH2-C-CH2COOH
H2O
CH3C-CH2COOH
Acetoacetate
Fumarylacetoacetate
HOOC-CH
HC-COOH
Fumarate

FIGURE 1

Metabolic Sequence from Tyrosine to Fumarate and Acetoacetate.

section). To a small extent, side paths of minor importance may occur in which p-hydroxyphenylpyruvate may be reduced to p-hydroxyphenyllactate or decarboxylated to p-hydroxyphenylacetate, neither of which is known to be metabolized any further.

The major pathway by which p-hydroxyphenylpyruvate is catabolized involves its transformation to 2,5-dihydroxyphenylacetate (homogentisate) with the utilization of one mole of molecular oxygen. In this step the reaction is catalyzed by p-hydroxyphenylpyruvate hydroxylase, an enzyme which has been shown to require ascorbic acid or one of the other reducing agents which can replace it, for example, 2,6-dichlorophenolindophenol and glutathione. These agents are capable of preventing inhibition of p-hydroxyphenylpyruvate hydroxylase by high concentrations of its own substrate. The enzyme is also inhibited by metal complex-forming agents, such as diethyldithiocarbamate and sodium azide, which is consistent with the hypothesis that p-hydroxyphenylpyruvate hydroxylase is a metal-containing enzyme. This enzyme is unique, since it apparently catalyzes three concerted reactions - ring hydroxylation and migration and oxidative decarboxylation of the side chain on the aromatic ring.

The "metabolic error", tyrosinosis, which may be "inborn" occurs at this site of the conversion of p-hydroxyphenylpyruvate to homogentisate. However, very few cases have been reported and very little is known of its heritability.

Homogentisate is in turn oxidized and its ring opened to form maleylacetoacetate, a reaction requiring utilization of another mole of molecular oxygen and the presence of ferrous iron, and catalyzed by homogentisate oxygenase. In this catabolic step reducing agents such as ascorbate are required to maintain the iron in the ferrous state and to suppress autoxidation of the substrate. The enzyme is inhibited by metal complex-forming agents, such as, ethylenediaminetetraacetic acid and α,α'-dipyridyl. Further catabolism of homogentisate is blocked in another inborn error of metabolism, "alcaptonuria", due to lack of the enzyme.

The product, maleylacetoacetate, is in turn acted upon by an enzyme, maleylacetoacetate isomerase, whose specific cofactor is glutathione. The product is fumarylacetoacetate and the reaction is a cis $\rightarrow$ trans isomerization.

Finally, fumarylacetoacetate is hydrolyzed by a specific hydrolase to one mole of fumarate and one mole of acetoacetate. The hydrolase requires no cofactors.

Since the pathway from tyrosine to fumarate and acetoacetate occurs entirely in the non-particulate fraction and has no side paths of any importance, it is of interest to determine whether the five enzymes involved are subject to similar developmental controls.

In the normal developing animal no intermediates accumulate in this pathway, suggesting co-ordinated rates of development. The exception occurs in the premature infant maintained on protein-rich diets, where the activity of tyrosine transaminase may exceed that of p-hydroxyphenylpyruvate hydroxylase.

HISTORICAL REVIEW

A. Regulation of Tyrosine Metabolism: Activation and Inhibition in the Adult Rat

1. TYROSINE TRANSAMINASE

a. Induction

Many investigations in the tyrosine pathway have been concerned with the postnatal and adult increase or decrease of the so-called "adaptive enzymes".

Injection of glucocorticoids resulted in as much as 10- to 20-fold adaptive increase in the activity of liver tyrosine transaminase in 5 hours in either intact or adrenalectomized rats. In contrast, the injection of the substrate of this enzyme, L-tyrosine, increased the enzyme level in the intact animals only, unless hydrocortisone was also administered. Tyrosine administered together with hydrocortisone produced a greater than additive increment of the enzyme activity in the adrenalectomized animal. It was concluded that the presence or release of glucocorticoid was a necessary condition for the enzyme level to respond adaptively to the substrate. Glucocorticoid was a sufficient stimulus, since tyrosine was ineffective in the absence of adrenal glands (2).

The increases of the protein moieties of tyrosine transaminase induced by hydrocortisone were not affected by a deficiency of the coenzyme, pyridoxal phosphate, in the animal (3). Apparent specificity for tyrosine and dependence on glucocorticoids suggested a dual control of this enzyme - one hormonal and the other a specific substrate adaptation. Reinvestigation of the tyrosine induced activation of the transaminase showed that the effect was non-specific and mediated entirely by adrenalcorticoid hormones (4). However, a specific tyrosine effect has been reported in young rats up to the age of 75 days (5).

Extension and refinement of earlier work, using immunological techniques to detect synthesis of enzyme protein, established that the increased transaminase activity following glucocorticoid administration was paralleled by an increase in immunologically detectable enzyme protein. Isotope studies led to the conclusion that induction increased the rate of enzyme synthesis and that alteration in the rate of degradation of the enzyme was not significant (6, 7, 8).

More recently, Greengard, Smith and Acs clearly distinguished between the two different mechanisms of induction by the use of inhibitors (9). Both inductions represented increased synthesis of enzyme protein, hence both were prevented by puromycin, an inhibitor of protein synthesis at the ribosomal level. Actinomycin D, an inhibitor of the DNA-dependent RNA synthesis, pre-

vented the glucocorticoid induction of tyrosine transaminase (as well as the postnatal rise of the enzyme in new-born rats), but did not prevent the induction of this enzyme by its substrate.

Paradoxically, actinomycin D at concentrations greater than 0.1 µg/ml or continuous treatment of young rats with the inhibitor over a period of 5 days caused an increase in tyrosine transaminase activity even after adrenalectomy (10). Very recently Tomkins and Peterkofsky (11) studied this effect in hepatoma cells in tissue culture to which cycloheximide (an inhibitor of protein synthesis at the ribosomal level) was added in the presence or absence of 2.0 µg actinomycin D per ml. They reported that, in the presence of cycloheximide, with or without actinomycin D, the level of tyrosine transaminase was decreased, suggesting that the antibiotic induction probably results from increased synthesis of the enzyme rather than activation or decrease in the rate of degradation.

Barnabei and Sereni (12) showed that adding 6-C^{14}-orotate to the perfusing blood of an isolated rat liver caused a rapid incorporation of radioactivity into nuclear ribonucleic acid to take place; cytoplasmic ribonucleic acid was poorly labelled after 30 minutes, but its activity rose thereafter. Cortisol stimulated the labelling of both the nuclear and cytoplasmic ribonucleic acid; moreover it induced an increase of tyrosine transaminase after a lag of about 30 minutes. In both cases,

the effect of the hormone was prevented by actinomycin D or by mitomycin C. These investigators found that livers perfused with cortisol had a higher nuclear RNA polymerase (nucleoside-triphosphate: RNA nucleotidyl transferase, EC 2.7.7.6) activity than the controls. The fact that the synthesis of nuclear ribonucleic acid preceded the increase of tyrosine transaminase and also that cortisol stimulated ribonucleic acid polymerase strongly suggested that the action of the hormone can be related to an increase in the synthesis of messenger ribonucleic acid molecules for the synthesis of the enzyme.

In more recent work there was isolated from the liver of cortisol-treated rats a mRNA fraction which when added to an *in vitro* protein-synthesizing system gave rise to tyrosine transaminase activity. The appearance of enzyme activity was prevented by puromycin and erythromycin, hence development of enzyme activity was dependent on active protein synthesis. It was concluded that the mRNA fraction contained the information for the formation of tyrosine transaminase and that the enzyme was formed, *in vitro*, by a "*de novo*" synthesis (13).

In contrast to the results obtained with slices (14) and homogenates (15), glucocorticoid induced an increase in tyrosine transaminase in the isolated, perfused rat liver, maintenance of the higher level being dependent on continuous provision of the hormone (16). Puromycin prevented the induction and lowered the basal level of the transaminase. Similar

induction in the perfused liver was observed in another laboratory, the most marked effect resulting from simultaneous provision of both hormone and tyrosine. Induction was blocked by anaerobiosis, chloramphenicol or ethionine (17).

Tyrosine-ketoglutarate transaminase activity was high in Morris hepatoma 5123 and was not significantly influenced by administration of tyrosine or glucocorticoid to the host, although the activity in the host liver rose markedly in response to that treatment. On the other hand, activity in tumors of adrenalectomized animals was lower than in intact animals and was raised by administration of glucocorticoids (18).

The apparent simultaneous induction of tyrosine-, phenylalanine-, and tryptophan-ketoglutarate transaminase in livers of rats treated with the respective amino acids or glucocorticoid was explained by the non-specificity of a single transaminase (19).

During the latter stages of growth of Walker carcinoma 256, a two-fold increase in liver tyrosine transaminase was noted, a change which was not seen in regenerating liver (20). Therefore, it seems unlikely that increased transaminase activity in the late stages of tumor growth resulted from an enhanced liver mitotic activity.

In adrenalectomized rats, the pancreatic hormones, insulin and glucagon, initiated a rapid but limited increase in the enzyme level, reaching a maximum after 3 hours (21). Immuno-

chemical-isotopic analyses showed that the pancreatic hormones caused an increase in the rate of synthesis of tyrosine transaminase comparable to that caused by hydrocortisone. Effects of the two pancreatic hormones were not additive with one another but were additive with the effect caused by hydrocortisone. Induction by either insulin or glucagon was inhibited by actinomycin D. The discrete steroid-independent induction of tyrosine transaminase by glucagon is of great interest for this is the only hormone shown so far to bring about an explosive accumulation of tyrosine transaminase prematurely by a single administration of the hormone to fetuses.

An additional mechanism for control of tyrosine transaminase level was proposed by Greengard and Gordon (22), who found that administration of large doses of pyridoxine caused three-fold elevation of this enzyme in 4 hours in the livers of adrenalectomized rats. Termed "cofactor induction", the increase in transaminase was reported to be preceded by increased hepatic levels of pyridoxal phosphate and to be prevented by puromycin but not by actinomycin D. This type of induction appeared to be analogous to the substrate induction of tryptophan pyrrolase by tryptophan (23, 24). Tryptophan stabilized tryptophan pyrrolase both *in vitro* and *in vivo* (25). Immunochemical analyses established that stabilization by the substrate brought about the increased pyrrolase level in adrenalectomized rats given tryptophan; that is, there was no change in the rate of enzyme synthesis (26).

Holten, Wicks and Kenney (27) reinvestigated the role of pyridoxine and its coenzymatically active derivative, pyridoxal phosphate, in determining the stability of rat liver tyrosine transaminase *in vitro*, and in addition tested the possibility that increased tyrosine transaminase levels following pyridoxine administration reflect stabilization of the transaminase by its coenzyme *in vivo*. They presented evidence that protection by coenzyme involved its reaction with the enzymatically active sites and that induction of tyrosine transaminase by pyridoxine administration did not reflect stabilization *in vivo*. Cofactor induction involved an increased rate of transaminase synthesis, determined immunochemically, and was inhibited by actinomycin D. It is thus similar to the inductions of this enzyme brought about by hydrocortisone and other hormones.

A number of indole derivatives, including L-tryptophan, D-tryptophan, acetyl-L-tryptophan, indole, DL-5-hydroxytryptophan and serotonin induced synthesis of tyrosine transaminase in livers of both normal and adrenalectomized rats (28). This was termed "substrate induction" and was prevented by ethionine and puromycin but not by actinomycin D. However, Labrie and Korner (29) showed that administration of amino acids to adrenalectomized rats stimulated the activity of tyrosine transaminase and tryptophan pyrrolase by a process that required RNA synthesis. The stimulation by tryptophan of tyrosine transaminase activity was entirely and that of the pyrrolase partially dependent on RNA synthesis. These findings

are contrary to the generally accepted view that the stimulation of activity of the enzymes by their substrates does not require RNA synthesis and thereby differs from the hydrocortisone stimulation.

Circadian fluctuation in the activity of hepatic tyrosine transaminase in rats maintained under normal laboratory conditions has been demonstrated (30) and confirmed (31, 32). Enzyme activity was almost four times as great several hours after nightfall as it was in the morning. Although glucocorticoid hormone concentrations in blood also vary rhythmically (2, 33), the enzyme rhythm was not produced by the hormone rhythm, inasmuch as it persisted in adrenalectomized or hypophysectomized animals (30).

Studies on the mechanism of the daily rhythm in hepatic tyrosine transaminase presented evidence that the rhythm was generated both by the tendency of the rat to consume most of its food during certain hours of the day and by the presence of amino acids, especially tryptophan, in the diet (34). The daily rhythm was rapidly extinguished when the rat was deprived of dietary protein and also when starved. It could be restored when the animal was given access to a mixture of amino acids, but only if this mixture included tryptophan. In the absence of other amino acids, dietary tryptophan alone could produce gross oscillations in tyrosine transaminase activity, provided that this amino acid was present in high concentrations.

Unfractionated calf thymus histones inhibited the induction of tyrosine transaminase in adrenalectomized mice when administered before the glucocorticoid inducer, but had an inducing action when given alone (35). In more recent work, decreasing order of inducing effectiveness was shown by polylysine, hydrocortisone, arginine-rich histone, lysine-rich histone, protamines, and unfractionated histones (36). Decreasing order of inhibition of hydrocortisone induction was shown by lysine-rich histone, arginine-rich histone, and unfractionated histones. Hydrocortisone induction was slightly augmented by protamine and polylysine.

In studies on the induction of another enzyme, tryptophan pyrrolase, injection of hydrocortisone was followed by an immediate decrease of liver nuclear histone concentration, reaching a minimum in 2-4 hours, then rising to the control level in 24 hours (37). Enzyme activity reached a maximum in 3-4 hours then fell to control levels in 24 hours. Histones were suggested as repressors of genetic activity by inhibition of mRNA synthesis.

Mason and Singer reported that benzoate caused a 4-fold increase in tyrosine transaminase activity in the livers of fasted adrenalectomized rats within 3 hours after intragastric administration (38). The effect was dose-dependent in the range of 5 to 20 mg per 150 gm body weight.. Of the 31 related compounds tested at equivalent dose levels, several (cyclohexanoate, p-aminobenzoate and α-naphthoate) were as effective as benzoate;

most were less effective or ineffective. Both actinomycin D and puromycin strongly inhibited the benzoate effect; in this regard the benzoate effect resembled that of the glucocorticoids.

Injection of reserpine induced an increase in tyrosine transaminase activity which exhibited a different time-course than that following glucocorticoid injection, in that very high levels of the enzyme were still observed after 72 hours and the enzyme activity returned to normal only after four days (39). The fact that tyrosine transaminase activity was still elevated when both the pharmacological and the neurohormonal effects of the alkaloid had disappeared, is not easily understandable.

Activation similar in characteristics to that due to glucocorticoids was produced by injection of total rat liver RNA, high molecular weight yeast RNA, commercial low molecular weight RNA and mixtures of 2',3'-nucleotides (40). The activation by RNA was dependent on the adrenal glands, but the dependence was not absolute in the case of the nucleotides.

Pre-incubation of a liver supernatant microsomal system from normal rats in the presence of Tris buffer, KCl, ATP, NAD, ketoglutarate, $MnCl_2$, glycine and $MgCl_2$ resulted in an increment of tyrosine transaminase activity (41). The system operated anaerobically, varied with temperature and pH, and was stimulated by NAD and 3',5'-cyclic-AMP. Puromycin and ethionine, RNase, or nuclear RNA had little effect. Since puromycin and ethionine do

not inhibit the observed increase in enzyme activity, the increment was not due to de novo synthesis of enzyme. It could arise in several ways, such as:

1) release of a bound form of the enzyme from the microsomal or soluble fractions or both,
2) an incubation-dependent activation of the apoenzyme,
3) an incubation-dependent removal of an inhibitor, or
4) a physical change of the protein to a more active state. However, direct evidence on these points is not yet available.

Cycloheximide has been reported to increase the level of tyrosine transaminase in rat liver (42). The level of the enzyme was higher when hydrocortisone was administered with cycloheximide, indicating an additive effect. In adrenalectomized rats, the antibiotic alone caused a large increase of tyrosine transaminase in 4 hours. This effect was inhibited by puromycin. The induction of tyrosine transaminase by cycloheximide has been disputed by other workers (43) who used rats anesthetized with pentobarbital and found that they were unable to confirm that effect in the anesthetized animal. Their data showed that cycloheximide inhibited the induction caused by hydrocortisone in adrenalectomized rats or by stress in intact rats.

In a new series of experiments (44) the induction of tyrosine transaminase by cycloheximide in adrenalectomized rats was confirmed, and it was found that pentobarbital abolished this increase. It was assumed that glucagon may have been responsible

for the increased tyrosine transaminase by cycloheximide and in accord with this assumption, cycloheximide was found not to increase tyrosine transaminase in pancreatectomized rats. It seems, therefore, that pentobarbital interferes with the release of glucagon by cycloheximide. In contrast to the results obtained with adult rats, Benson and Young (45) reported that administration of cycloheximide to newborn rats consistently produced partial inhibition of developmental increase of tyrosine transaminase activity.

b. Repression

Rivlin and Levine (46) reported that 4-week periods of thyroxine administration to rats with intact adrenal glands increased the activity of tyrosine transaminase by 40%, whereas thyroidectomy reduced activity by 50%. The studies that they carried out later were designed to determine whether thyroxine potentiated the action of hydrocortisone as an inducer of tyrosine transaminase. The results demonstrated instead that thyroxine treatment tends to minimize the effectiveness of hydrocortisone as an inducer, whereas thyroidectomy tends to enhance its effectiveness. Experiments demonstrated that the induction of tyrosine transaminase in rat liver by hydrocortisone can be modified by changes in the thyroid status of the animal. A given dose of hydrocortisone was a more effective inducer in hypothyroid animals than in either euthyroid

or hyperthyroid animals; by contrast, in hyperthyroid animals hydrocortisone was entirely without effect unless a massive dose was given.

Evidence for a mechanism by means of which thyroxine treatment minimized hydrocortisone induction was obtained from subsequent studies involving the hepatic uptake of radioactive hydrocortisone and measurement of the amounts of steroid degraded (47)[1]. The ratio of metabolites of hydrocortisone to hydrocortisone was 3 times greater in livers from hyperthyroid animals than in livers from euthyroid animals. These findings are consistent with those of previous investigators, who have shown increased capacity for degradation of corticosteroids by livers from hyperthyroid animals (48-50). These data demonstrated that, in hyperthyroid animals, whatever steroid was available to induce tyrosine transaminase was being metabolized much more rapidly than in control animals. It was concluded that thyroxine minimized the action of hydrocortisone as an inducer of hepatic tyrosine transaminase by increasing the rate of steroid degradation.

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[1] It is important to note that Goldstein, Stella, and Knox (16) had previously shown that, in isolated, perfused rat liver, tyrosine transaminase (but not tryptophan pyrrolase) could be maintained in the induced state only when a continuous supply of hydrocortisone was present in the blood circulating through the liver. One would expect, therefore, that hydrocortisone would not exert a maximal and sustained effect in any condition which tended to accelerate its rate of degradation.

Kenney (51) reported that moderate doses of the hypophyseal growth hormone could effect a selective repression of the synthesis of rat liver tyrosine transaminase in hypophysectomized, as well as in adrenalectomized, rats. Treatment with growth hormone brought about a transitory reduction in the level of this enzyme in the liver. This reduction was due to a marked, and sometimes complete, cessation of synthesis of the enzyme, as shown by direct isotopic analysis and also by virtual identity of the rate of reduction with the known rate of degradative removal of the enzyme in normal turnover, while labelling of the bulk of the liver proteins was increased by the hormone. The inhibition of the synthesis of tyrosine transaminase was, therefore, selective.

This repression is, however, apparently not entirely unique to this enzyme, for Gaebler (52) and Harding and Rosen (53) have described a similar reduction in the glutamic-alanine transaminase (also inducible by glucocorticoids) in the livers of growth hormone-treated rats. However, tryptophan pyrrolase levels were stable in the livers of stressed rats in which tyrosine transaminase was repressed (54), so it would seem that repression by growth hormone is not common to all the glucocorticoid-inducible enzymes.

It should be noted, however, that the response to growth hormone was erratic and the basis for the inconstancy has not yet been identified. One possibility is the competing action of inducing hormones not removed by adrenalectomy, such as insulin and glucagon. Under some circumstances, treatment with growth hormone may result in the release of insulin or glucagon, or both, in quantities to block repression; the increase in enzyme sometimes seen to precede repression could be due to an effect of this kind. Furthermore, in preliminary studies with isolated perfused livers and with cultures of rat hepatomas (55, 56), growth hormone failed to effect this repression. Thus it is quite possible that repression of hepatic enzyme synthesis is secondary to some other metabolic change brought about by growth hormone *in vivo*; such a secondary response might be expected to be erratic. Further experimentation is clearly needed to resolve this question.

2. p-HYDROXYPHENYLPYRUVATE HYDROXYLASE

p-Hydroxyphenylpyruvate hydroxylase levels have not undergone changes in parallel with those of tyrosine transaminase brought about by inducing agents (glucocorticoids, amino acids) (57).

Injection of p-hydroxyphenylpyruvate into scorbutic guinea pigs inhibited over half of the hydroxylase activity in the liver, but this could be reactivated, *in vitro*, by ascorbic

acid or reduced dichlorophenolindophenol (58, 59). Livers of scorbutic animals contained the same concentration of the hydroxylase as normal livers. Ascorbic acid and dichlorophenolindophenol prevented inhibition of the enzyme by high concentrations of its own substrate.

Excretion of p-hydroxyphenylpyruvate on feeding tyrosine to normal or scorbutic guinea pigs was a result of induction of tyrosine transaminase coupled with substrate-depression of p-hydroxyphenylpyruvate hydroxylase, the latter change being reversed *in vivo* or *in vitro* by ascorbate or dichlorophenolindophenol (60).

p-Hydroxyphenylpyruvate hydroxylase was shown to be nearly twice as sensitive to protein deprivation as tyrosine transaminase, (the enzyme activity being reduced by about 85% within seven days) and was slightly less rapidly restored by protein elevation (61).

The inactive form of the hydroxylase found in liver of tyrosine-fed animals was produced by subjecting the enzyme, *in vitro*, to various treatments, predominantly oxidizing or metal-chelating agents (62). Inhibition by various sulfhydryl reagents and reversal of mercuric ion inhibition by glutathione (reduced) indicated that the enzyme contained essential sulfhydryl groups. The requirement for reduced glutathione for optimal activity of the enzyme in the presence of dichloro-

phenolindophenol was attributed to the protection of the sulfhydryl groups on the protein molecule. It was also suggested that activation by ascorbate or dichlorophenolindophenol was non-specific and involved re-formation or maintenance of an essential metal group in the reduced form (63).

In the young rat fed tyrosine on a purified diet, tyrosyluria was caused by the usual induction of the tyrosine transaminase, but the concomitant slight inactivation of the hydroxylase due to tyrosine, which was reversible in vitro, was not prevented by administration of ascorbate in vivo (5). In addition, the major part of the depression in hydroxylase activity was caused by the purified diet, which, while it did not decrease the amount of enzyme already in the liver, inhibited further synthesis (but only in the rapidly-growing, young animal), possibly due to lack of some necessary factor in the diet.

Most of the depression in over-all tyrosine oxidation seen in livers of thyroid-treated animals has been attributed to reduction in the activity of p-hydroxyphenylpyruvate hydroxylase (64).

3. HOMOGENTISATE OXYGENASE

No systematic study of the regulation of this enzyme has been made. However, the oxygenase was unaffected by protein deprivation (61), was lower than normal in rats made alcapto-

nuric by a high tyrosine diet (65), and could be increased adaptively by administration of tyrosine or glucocorticoid (57). The reports are contradictory, pointing out the need for further experimentation to sort out the mechanisms operating in the control of this enzyme.

4. MALEYLACETOACETATE ISOMERASE AND FUMARYLACETOACETATE HYDROLASE

It was suggested that these enzymes are constitutive, not adaptive to substrate, since they were found in normal concentration in the liver of alcaptonurics (66). However, no systematic study of their control has been carried out.

B. Developmental Studies

1. TYROSINE TRANSAMINASE

It has been reported that ACTH and, less consistently, cortisone decreased the tyrosyluria of premature infants on high protein, scorbutogenic diets (67). ACTH had a variable and transient effect in decreasing excretion of keto acids and tyrosyl compounds in scorbutic guinea pigs, while corticoids had no effect (68). The difference in action between the pituitary hormone and the adrenal hormones seems to be the apparent ability of ACTH to mobilize tissue stores of ascorbate, thus making it available for metabolic process in the organism.

The over-all rate of oxidation of tyrosine in the liver of premature infants was found to be consistently less than

since it is known (74) that a single injection of this drug induces adrenal insufficiency for only a limited time. When added directly to the assay system in vitro, Amphenone had no effect on the tyrosine transaminase activity of liver homogenates, a finding which supported the assumption that the drug acts indirectly by inhibiting, at least partially, the formation of adrenal steroids. That the adrenal glands do, indeed, play an important part in the development of the transaminase was confirmed by finding that adrenalectomy at birth caused a marked inhibition of this development, an inhibition which can be reversed by administration of hydrocortisone.

In rabbit, guinea pig, and chicken, the phenomenon of birth (or hatching) appeared to make the liver responsive to the induction of tyrosine transaminase by glucocorticoids (75).

The large increase in transaminase activity after birth, as in the activation by glucocorticoids, reflected an increase in immunologically antigenic protein detectable by reaction with antisera to the purified enzyme, suggesting that in both cases the increase in enzyme activity was due to de novo apoenzyme synthesis (6-8).

Tyrosine transaminase activity increased in the maternal liver of pregnant rats from the 16th to the 19th day, then dropped to the normal levels of non-pregnant rats (76). It rose in response to glucocorticoids, protein-rich food,

epinephrine injection, and administration of tyrosine and fell after protein-free food and adrenalectomy. The embryonic liver had low activity which remained relatively unaffected during the various treatments of the mother. Sereni et al. (72) were also unable to detect any significant effect of hydrocortisone when fetuses were exposed to the hormone for 5 hours, a result consistent with the data obtained by Yeung and his co-workers (77), who did, however, find that tyrosine transaminase activity in fetal rat liver was markedly elevated in animals exposed to the hormone for 10 hours or more, but only in animals close to term.

The increase in tyrosine transaminase activity shown after prolonged exposure suggests that the hormone was reaching a target system in the liver, whatever the precise nature of the system might be. The results also indicate that glucocorticoids do not initiate the synthesis of this enzyme, since it has not been possible to cause tyrosine transaminase to appear prematurely by the prenatal administration of glucocorticoid to fetuses that are not close to term. The glucocorticoid may be a necessary but not a sufficient factor in this developmental step. It is suggested that such hormones play a part in control mechanisms for regulation of enzyme activities once synthesis is initiated.

The observation that induction of liver tyrosine transaminase by hydrocortisone in adult, adrenalectomized rats was greatly enhanced by prior starvation (78) provided the first indication of an unknown factor that may regulate the level of this enzyme.

Later experiments on adult rats (79) provided a lead to another hormonal factor that was involved in the development of tyrosine transaminase. The abrupt accumulation of tyrosine transaminase that normally occurs in rats at birth has been brought about prematurely by a single administration of glucagon to fetuses. Serine dehydratase, which accumulates normally after birth, and glucose-6-phosphatase, which begins to develop just before birth, have been prematurely increased in fetuses by glucagon. These findings suggest that glucagon may exert an important physiological function in the rat during the time of major developmental change in carbohydrate and amino acid metabolism.

An injection of glucagon to fed rats reproduced the effect of fasting on tyrosine transaminase (79). The possibility that the same factor might be involved in the developmental formation of tyrosine transaminase was considered, because this enzyme appears after birth, shortly after the cessation of the placental supply of nutrients. Two hours after birth the blood glucose level reaches a minimum of 20 mg/100 ml (80), and the secretion of glucagon is known to be inversely related to the level of blood glucose (81).

The administration of glucose inhibits the early postnatal accumulation of tyrosine transaminase and serine dehydratase during the first 6 to 8 hours after birth. The results are consistent with the possibility that, under normal conditions, the

decrease in blood glucose, upon cessation of the placental supply at birth, stimulates the secretion of glucagon, which then triggers the formation of tyrosine transaminase and serine dehydratase (21).

Epinephrine at concentrations of 10^{-6} M or higher produced a two- to three-fold increase in the tyrosine transaminase activity in explants of fetal rat liver maintained in organ culture. Experimental results were consistent with the conclusion that epinephrine induces tyrosine transaminase by a mechanism involving cyclic AMP, and that this mechanism is different from that by which hydrocortisone increases the synthesis of this enzyme (82). Since glucagon also increases the concentration of cyclic AMP in the liver, it seems reasonable to suppose that induction by this hormone should proceed via cyclic AMP as an intermediate. However, the response to insulin induction is virtually identical to that with glucagon, in vivo (83), and yet insulin lowers the concentration of cyclic AMP in the liver (84). This apparent discrepancy points out the need for further experimentation to resolve the mechanisms operating to control the synthesis of tyrosine transaminase.

2. p-HYDROXYPHENYLPYRUVATE HYDROXYLASE

Livers of premature infants lacked p-hydroxyphenylpyruvate hydroxylase activity, part of which could be restored by in vitro addition of ascorbate (85).

Livers of fetal rats and guinea pigs had lower basal levels of hydroxylase activity than adult livers, but rose to adult levels after birth, slowly in the rat and rapidly in the guinea pig (86, 87). Fetal, but not adult, enzyme activity could be raised several fold by preincubation with dichlorophenolindophenol and glutathione. The activatable fraction in fetal livers resembles that which occurs in adult guinea pigs after tyrosine feeding.

3. HOMOGENTISATE OXYGENASE

The metabolic abnormality in alcaptonuria was scarcely noted 38 hours after birth, although after 52 hours it was evident and continued thereafter (88) in an alcaptonuric infant. Other developmental studies of this enzyme are lacking.

4. MALEYLACETOACETATE ISOMERASE AND FUMARYLACETOACETATE HYDROLASE

No systematic studies of these enzymes have been made during development.

EXPERIMENTAL PROCEDURES

A. Sources of Materials and General Procedures

1. MATERIALS

Pregnant Wistar rats from West Jersey Biological Farms were kept in the animal room and watched closely until they delivered. Compounds of highest available purity were obtained from a variety of commercial sources. L-Tyrosine, potassium chloride, potassium phosphate (monobasic), ammonium molybdate, disodium ethylenediaminetetraacetate, p-hydroxyphenylpyruvic acid, sodium-α-ketoglutarate, pyridoxal phosphate, reduced glutathione, L-ascorbic acid and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Company. Sodium 2,6-dichlorophenolindophenol, sodium diethyldithiocarbamate and N-ethylmaleimide were obtained from Eastman Kodak Company. Homogentisic acid obtained from Sigma Chemical Company was recrystallized before use. Meta-phosphoric acid was obtained from J. T. Baker Chemical Company and crystalline bovine serum albumin was obtained from Mann Laboratories. Filters were obtained from Millipore Filter Company.

2. PREPARATION OF TISSUE EXTRACTS

Pregnant rats were provided with Purina rat chow and water and the exact time of delivery was recorded by close observation. Body weights of the newborn animals were measured. Animals were killed by decapitation. The livers were removed as quickly as possible, pooled, maintained cold, weighed and rapidly homogenized (Potter-Elvehjem) in an ice bath with four parts (weight per volume) of ice-cold 0.14M KCl-0.02M phosphate buffer, pH 7.6. The homogenates were then centrifuged in the cold room in a Sorval centrifuge Model SS-1 at 9,000 x g for one hour. The clear supernatant fractions were stored at -20° for the assay of all five enzymes and estimation of protein. All such determinations were carried out in duplicate.

3. PROTEIN ESTIMATION

Proteins were estimated by the biuret method (89). One milliliter of supernatant fraction was pipetted into a conical-bottom centrifuge tube and to it 4.0 ml biuret reagent were added and mixed well. The mixture was allowed to stand for 30 minutes with stirring at frequent intervals. At this point the solutions were usually cloudy. Three milliliters of petroleum ether were added, the tube was vigorously shaken and then centrifuged to separate the layers. By this process lipid material was accumulated as a scum at the interface and could be removed, along with the petroleum ether with a Pasteur pipette. Extractions were repeated until the purple biuret-

protein solution was clear. To remove the last traces of lipid scum, the solution was forced through a 0.45 μ white plain Millipore filter. The absorbance was measured at 540 mμ in a Beckman B spectrophotometer against a blank mixture of biuret reagent and water. The protein content was calculated by comparison with a standard of crystalline bovine serum albumin.

4. PREPARATION OF MALEYLACETOACETATE

Since the substrates for maleylacetoacetate isomerase and fumarylacetoacetate hydrolase are not available commercially, it was necessary to carry out their preparations ourselves. Maleylacetoacetate is the primary product of homogentisate oxidation in animal liver and is apparently formed by the single-step addition of oxygen to homogentisate by homogentisate oxygenase.

Maleylacetoacetate has not been synthesized chemically, but solutions of it have been prepared enzymatically by homogentisate oxidation (90). The enzyme preparation used for this purpose was a supernatant fraction of a 20% rat liver homogenate in 0.14 M KCl-0.02 M phosphate buffer, pH 7.6, which was dialyzed exhaustively against a large volume of buffer in the cold room for 24 hours with efficient stirring and frequent change of buffer. The dialyzed liver extract was used to form maleylacetoacetate by the complete oxidation of homogentisate in the presence of 10 μmoles ascorbate, 180 μmoles

tris(hydroxymethyl)aminomethane buffer, pH 7.4, and 2.0 μmoles ferrous iron. The reaction was carried out in Warburg vessels with air as the gas phase. 0.5 ml of enzyme supernatant was used per vessel to catalyze the oxidation of 5 μmoles of homogentisate. When the oxygen uptake of the reaction stopped, the flasks were promptly chilled. The pooled contents of the experimental flasks were deproteinized with 0.1 volume of 20% metaphosphoric acid. The filtrates were adjusted to pH 8.0 and lyophilized. Fresh, dry preparations contained very little or no fumarylacetoacetate and were much more stable than the aqueous solutions.

5. PREPARATION OF FUMARYLACETOACETATE

The dry preparation described above was dissolved in a minimum volume of 1.0 N HCl, the pH adjusted to 1.0 or below, filtered, and refrigerated for one week to allow non-enzymatic acid isomerization of maleylacetoacetate to fumarylacetoacetate. This type of preparation contains considerable amounts of maleylacetoacetate.

6. DETERMINATION OF MALEYLACETOACETATE AND FUMARYLACETOACETATE IN MIXTURES OF THE TWO

Both these compounds were determined in the Beckman B spectrophotometer from their absorbances at pH 1.0 and 13.0. Maleylacetoacetate absorbs strongly only in alkaline solution, while fumarylacetoacetate absorbs strongly in both acid and alkaline

solutions at 330 mμ. These compounds can be determined singly or in mixtures (90, 91) by solving two simultaneous equations and two unknowns:

Maleylacetoacetate (μmoles per ml) = $A_{13}-A_1/14.8$ and

Fumarylacetoacetate (μmoles per ml) = $0.0801\ A_1 - 0.00601\ A_{13}$,

where A_1 and A_{13} are the observed absorbances at 330 mμ in 1.0 cm cells in 0.1 N HCl and 0.1 N NaOH respectively.

B. Enzyme Assays

1. ASSAY OF TYROSINE TRANSAMINASE

Tyrosine transaminase was routinely assayed by incubation of 0.1 ml liver extract for twenty minutes at 37° with 12 μmoles of L-tyrosine, 0.1 μmoles of pyridoxal phosphate, 30 μmoles α-ketoglutarate, 100 μmoles phosphate buffer, pH 7.6, and water to a final volume of 3.0 ml (composition of mixture slightly modified from that of Ref. 92). All components except α-ketoglutarate were pre-incubated for 5 minutes before the reaction was initiated by its addition. In the case of the zero-time samples, protein was precipitated before the addition of α-ketoglutarate. In this assay using unfractionated liver preparations, 5.0 μmoles of diethyldithiocarbamate were added, which effectively prevented product removal by p-hydroxyphenylpyruvate hydroxylase (93).

The reaction was stopped by the addition of 0.2 ml of 100% trichloroacetic acid and the denatured proteins removed by centrifugation, after allowing the reaction mixtures to stand at room temperature for 30 minutes. 2.5 ml aliquots of the clear

supernatant fractions were analyzed for p-hydroxyphenylpyruvate by the modified Briggs method (94), involving the addition of 2.0 ml of "mixed reagent", a freshly prepared 1:1 mixture of 3% ammonium molybdate in 5.0 N HCl and 1% aqueous potassium phosphate. The absorbance of the resulting colored solution was read after 1 hour on a Beckman Model B spectrophotometer at 770 mμ. The concentration of p-hydroxyphenylpyruvate was then calculated by comparison against the absorbance of a standard containing 0.2 μmoles of p-hydroxyphenylpyruvate carried through the assay procedure without the addition of liver extract, cofactors and α-ketoglutarate. The rate of formation of p-hydroxyphenylpyruvate was then calculated from the differences in concentrations of this compound between the zero-time and the incubated samples.

2. ASSAY OF p-HYDROXYPHENYLPYRUVATE HYDROXYLASE

The assay of p-hydroxyphenylpyruvate hydroxylase was carried out manometrically in a Warburg apparatus in which the main compartment contained 140 μmoles sodium phosphate buffer, pH 7.0, 1.0 μmole 2,6-dichlorophenolindophenol, 4.0 μmoles disodium ethylenediaminetetraacetate, 40.0 μmoles glutathione and 0.5 ml liver extract. The side arm contained 5.0 μmoles p-hydroxyphenylpyruvate in the experimental flasks and water in the control flasks. The center well contained 0.2 ml of 20% KOH and a filter paper wick. The total fluid volume in the main space was 3.0 ml. After equilibration at

37.5° for 10 minutes, the contents of the side arm were tipped in to start the reaction. Air was used as the gas phase.

The above procedure was adopted from Zannoni and La Du (95) but with the following modifications:

1. Disodium ethylenediaminetetraacetate (96) instead of α,α'-dipyridyl (97) was added to prevent further oxidation of homogentisate by inhibiting homogentisate oxygenase.

2. Sodium 2,6-dichlorophenolindophenol replaced ascorbate as the reducing agent capable of preventing an inhibition of p-hydroxyphenylpyruvate hydroxylase by high concentrations of substrate (98-100).

The first alteration was made on the basis of a few observations in the literature and our own data, indicating slight but measurable inhibition of the hydroxylase by dipyridyl, as compared with ethylenediaminetetraacetate (Table 2). The second change was prompted by reports of the superior effectiveness of the indophenol over ascorbate as enzyme "activator", confirmed by our data (Table 2).

Even under our optimum conditions, amounts of substrate greater than 5 μmoles per flask began to exhibit inhibition of the hydroxylase well before the 15 minute period used in the assay (Figure 2).

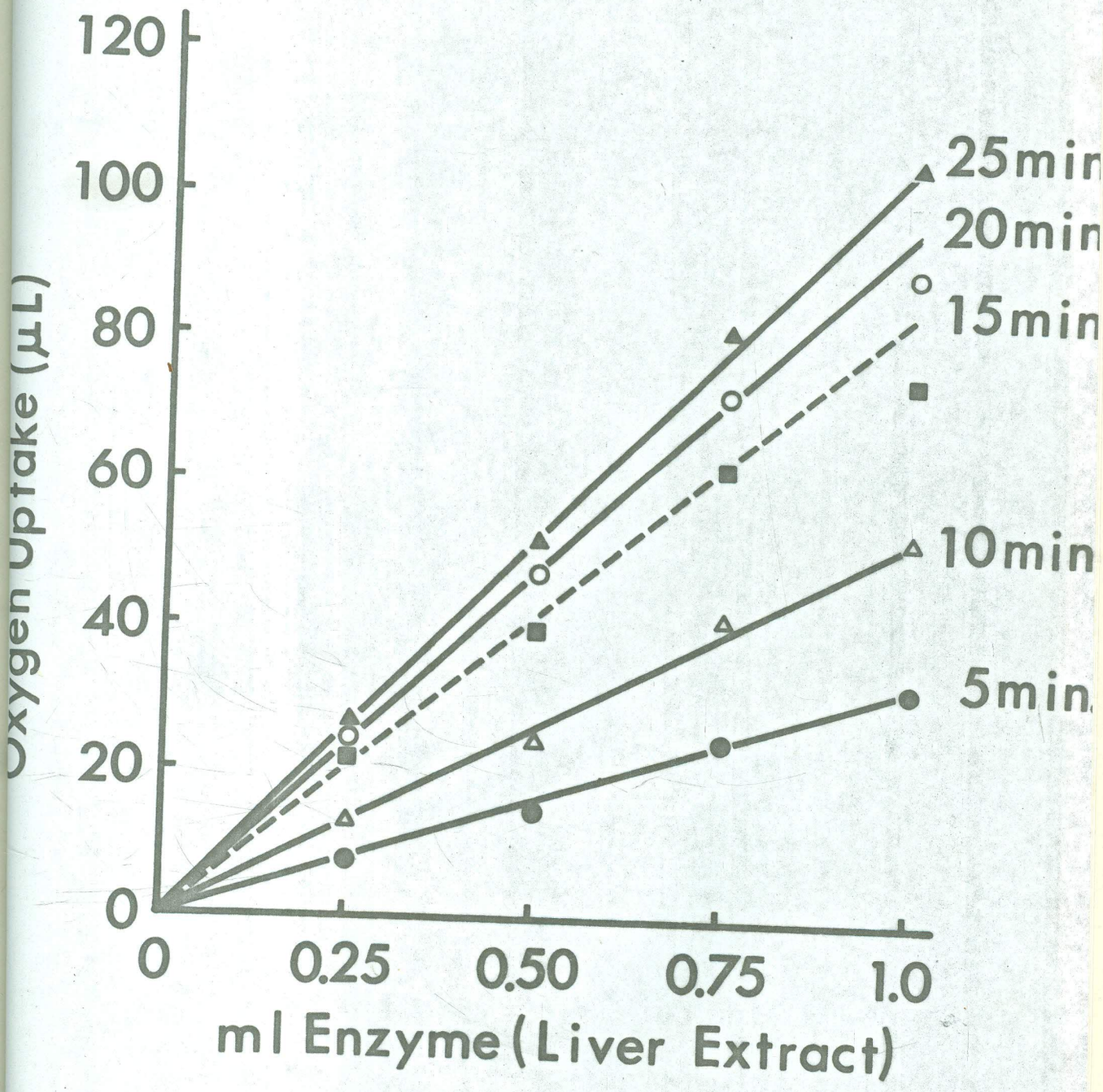
Since two major factors in the Zannoni-La Du procedure were altered, it was important to establish that proportionality was retained between concentration of enzyme and uptake of

TABLE 2

Effects of Various Combinations of Reducing and Chelating Agents on Oxidation of p-Hydroxyphenylpyruvate and Homogentisate

Flask Contents	Cumulative µL Oxygen Uptake at Indicated Times (min)								
	5	10	15	20	25	30	35	40	45
5.0 µmoles p-Hydroxyphenylpyruvate +									
1. 20.0 µmoles Ascorbate and 4.0 µmoles Dipyridyl added	24	41	52	63	69	75			
2. 1.0 µmole 2,6-DCPP and 4.0 µmoles Dipyridyl added	23	45	63	75	83	89			
3. 8.0 µmoles Ascorbate and 4.0 µmoles EDTA added	19	35	44	54	61	69			
4. 1.0 µmole 2,6-DCPP and 4.0 µmoles EDTA added	19	44	75	90	95	98	101	102	105
5.0 µmoles Homogentisate +									
1. 1.0 µmole 2,6-DCPP and 4.0 µmoles EDTA added	0	1	2	4	6	8	8	11	11

Figure 3


120
100
80
60
40
20
0
Oxygen Uptake (µL)
25min
20min
15min
10min
5min
0
0.25
0.50
0.75
1.0
ml Enzyme (Liver Extract)

oxygen. That this was so can be seen in Figure 3. The actual assay procedure utilized the oxygen uptake over a ten-minute period, extending from the 5- to the 15-minute reading. A re-plot of the data from Figure 3 on this basis is shown in Figure 4.

3. ASSAY OF HOMOGENTISATE OXYGENASE

The enzyme assay was carried out manometrically in Warburg vessels according to Ravdin and Crandall (91), with some modifications. Tris(hydroxymethyl)aminomethane buffer was used instead of 2,4,6-collidine buffer. Ascorbate was completely eliminated from the reaction mixture, since it has been shown that systems in which tris(hydroxymethyl)aminomethane serves as the buffer have a relatively high soluble iron level in the absence of ascorbate and essentially no requirement for ascorbate (101). Furthermore, the presence of ascorbate introduces very high oxygen uptake in the control flasks (Table 3). It is probable that ascorbate in the presence of homogentisate blocks the oxidation of the latter, or the presence of homogentisate may depress the oxidation of ascorbate. In either case, subtracting the ascorbate blank would lead to inaccurate results. The main compartment contained 190 µmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, 2.0 µmoles ferrous sulfate, 6.0 µmoles glutathione and 0.5 ml of liver supernatant fraction in 3.0 ml total volume. The side arm of the experimental flasks contained

Figure 2

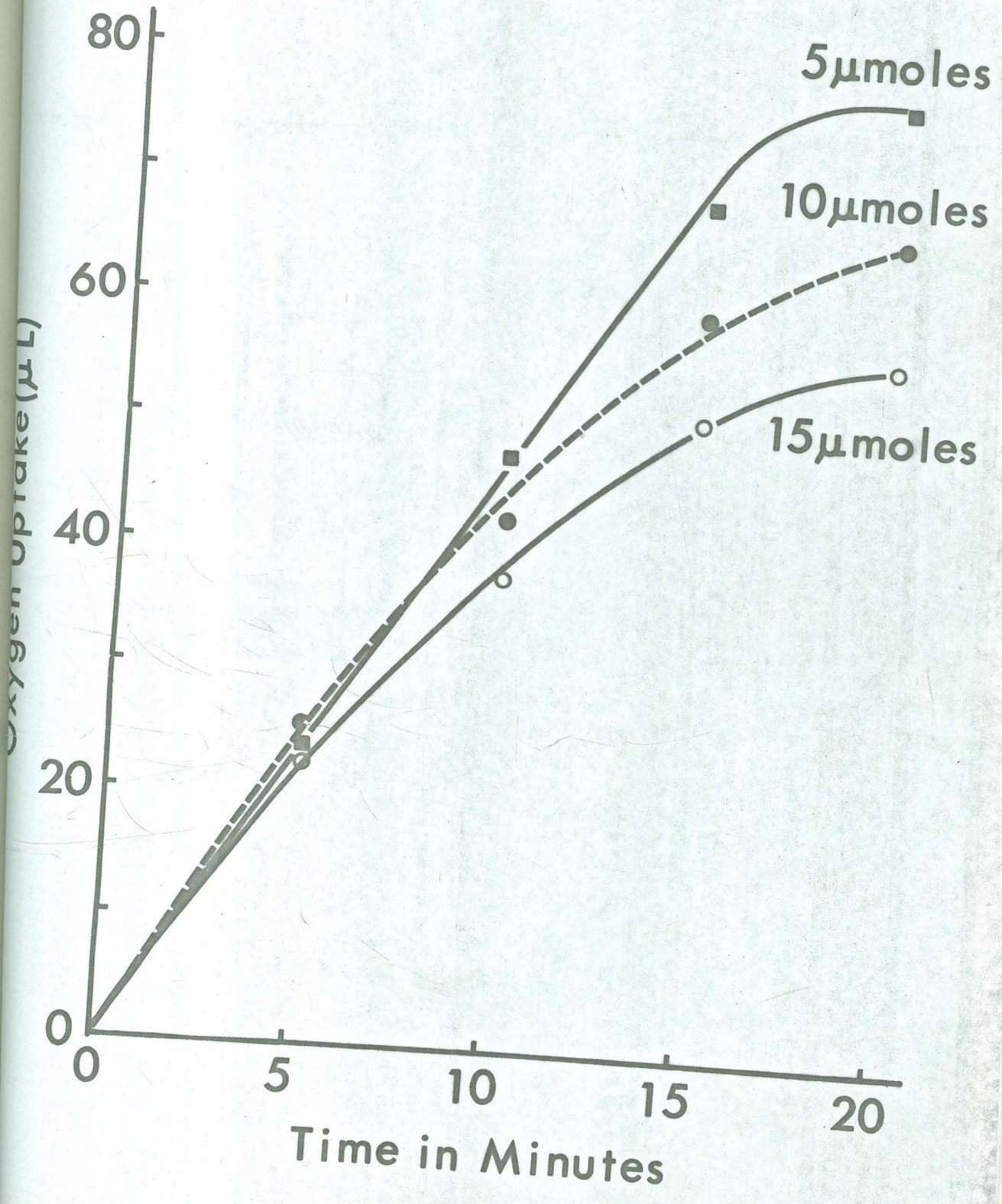

80
60
40
20
0
0
5
10
15
20
Oxygen Uptake (µL)
Time in Minutes
5µmoles
10µmoles
15µmoles

FIGURE 3

Linearity of Oxygen Uptake with Varying Concentrations of p-Hydroxyphenylpyruvate Hydroxylase.

The Warburg flasks contained 0.25 to 1.0 ml enzyme preparation, 140 μmoles phosphate buffer, pH 7.0, 40 μmoles glutathione, 1.0 μmole 2,6-dichlorophenolindophenol, 4.0 μmoles disodium ethylenediaminetetraacetate, and 5.0 μmoles of the substrate in a total volume of 3.0 ml. Control flasks contained no substrate. Gas phase was air, and temperature was 37.5°.

Figure 4

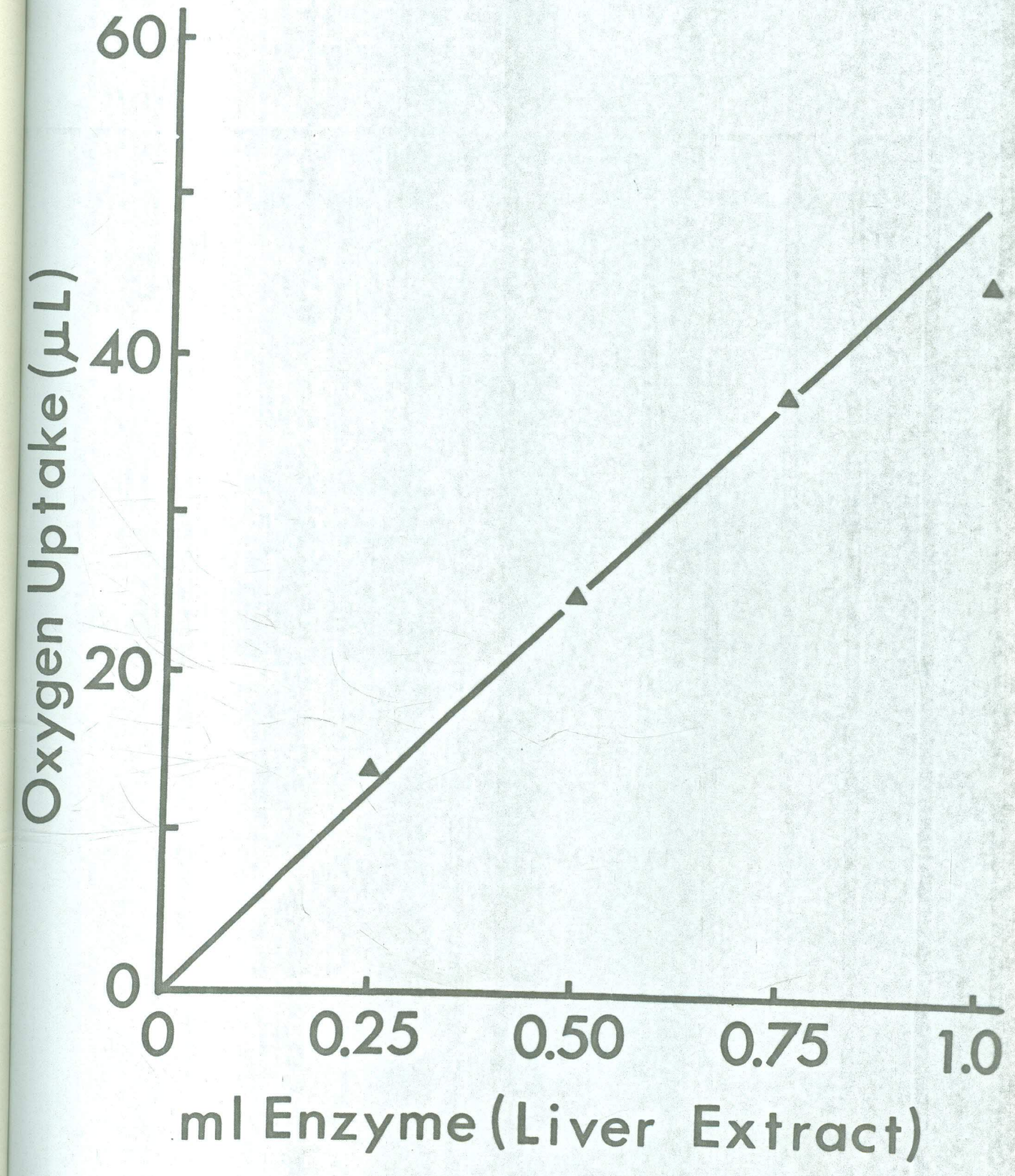

60
40
20
0
Oxygen Uptake (µL)
0
0.25
0.50
0.75
1.0
ml Enzyme (Liver Extract)

FIGURE 4

Manometric Assay of p-Hydroxyphenylpyruvate Hydroxylase.

A re-plot of the data from Figure 3, utilizing as ordinate the differences between the 5- and 15-minute readings.

TABLE 3

OXIDATION OF HOMOGENTISATE IN THE PRESENCE AND ABSENCE OF ASCORBATE

Time (min.)	Blank		Homogentisate added			
	Ascorbate added	No Ascorbate	Ascorbate added	No Ascorbate	Ascorbate added	No Ascorbate
	Oxygen uptake, µL					
5	9	1	24	20	25	16
10	20	1	47	43	49	37
15	32	0	70	65	72	60
20	42	1	92	89	96	82

TABLE 3

Oxidation of Homogentisate in the Presence and Absence of Ascorbate.

The Warburg flasks contained 190 µmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, 2.0 µmoles ferrous sulfate, 6.0 µmoles glutathione and 0.5 ml liver supernatant fraction in a total volume of 3.0 ml. Where indicated, flasks contained 5 µmoles homogentisate and/or 8 µmoles ascorbate.

5.0 μmoles homogentisic acid, while the substrate was omitted from the control flasks. The center well contained 0.2 ml of 20% KOH and a filter paper wick. After equilibration at 37.5° for 10 minutes, the contents of the side arm were tipped in to start the reaction. Air was used as the gas phase.

Glutathione was added to prevent non-enzymatic oxidation of homogentisate and to maintain the iron in the ferrous state (99).

4. ASSAY OF MALEYLACETOACETATE ISOMERASE

For assay of this enzyme, the continuous spectrophotometric method of Edwards and Knox (102) was changed to a fixed-time method, with some further modifications. Tris(hydroxymethyl)-aminomethane buffer, pH 8.75 was used instead of phosphate buffer, pH 7.5 (the optimum pH for the hydrolase, which is required in the Edwards and Knox method) and no isomerase-free hydrolase was added to the reaction mixture. The incubation mixture consisted of 50 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.75, 2.5 μmoles of glutathione (this was diluted daily from an unneutralized 0.1 M stock solution kept frozen), 0.1 ml 20% liver supernatant fraction, an amount of neutralized substrate preparation which would have an initial absorbance in the more sensitive range of the spectrophotometer (usually 1.0 ml) and water to a total volume of 3.0 ml. Mixtures for the blank were made up similarly, but with no substrate. The zero-time sample (1.0 ml) was pipetted out of the reaction mixture before the addi-

tion of the substrate and deproteinized with 1.0 ml of 20% meta-phosphoric acid. After standing a minimum of 15 minutes, one-third of the amount of substrate to be used for the assay was then added.

The remaining reaction mixture was equilibrated at 37° for 10 minutes, and at zero time the remaining amount of substrate was added and stirred well. After 3 minutes incubation a 1.0 ml aliquot was pipetted out and immediately deproteinized with 1.0 ml of 20% meta-phosphoric acid. The remaining 1.0 ml of the incubation mixture was allowed to incubate for 2 more minutes after which 1.0 ml of 20% meta-phosphoric acid was added to stop the reaction. This was the 5-minute sample.

All the tubes were allowed to stand at room temperature for 15 minutes, then centrifuged, and 0.5 ml aliquots of the clear supernatants were used for analysis of maleylacetoacetate and fumarylacetoacetate as indicated in the section dealing with the determination of these compounds (Section III. A. 6.) In the course of establishing the method for the assay of this enzyme, we were able to confirm the finding by Knox and Edwards (103) that this enzyme follows first-order kinetics (Figure 5). We therefore followed the practice of Knox and Edwards in expressing the activity of this enzyme in terms of the first-order reaction rate constant.

5. ASSAY OF FUMARYLACETOACETATE HYDROLASE

The general procedures for the assay of this enzyme were similar to those described for the isomerase. The substrate,

Figure 5

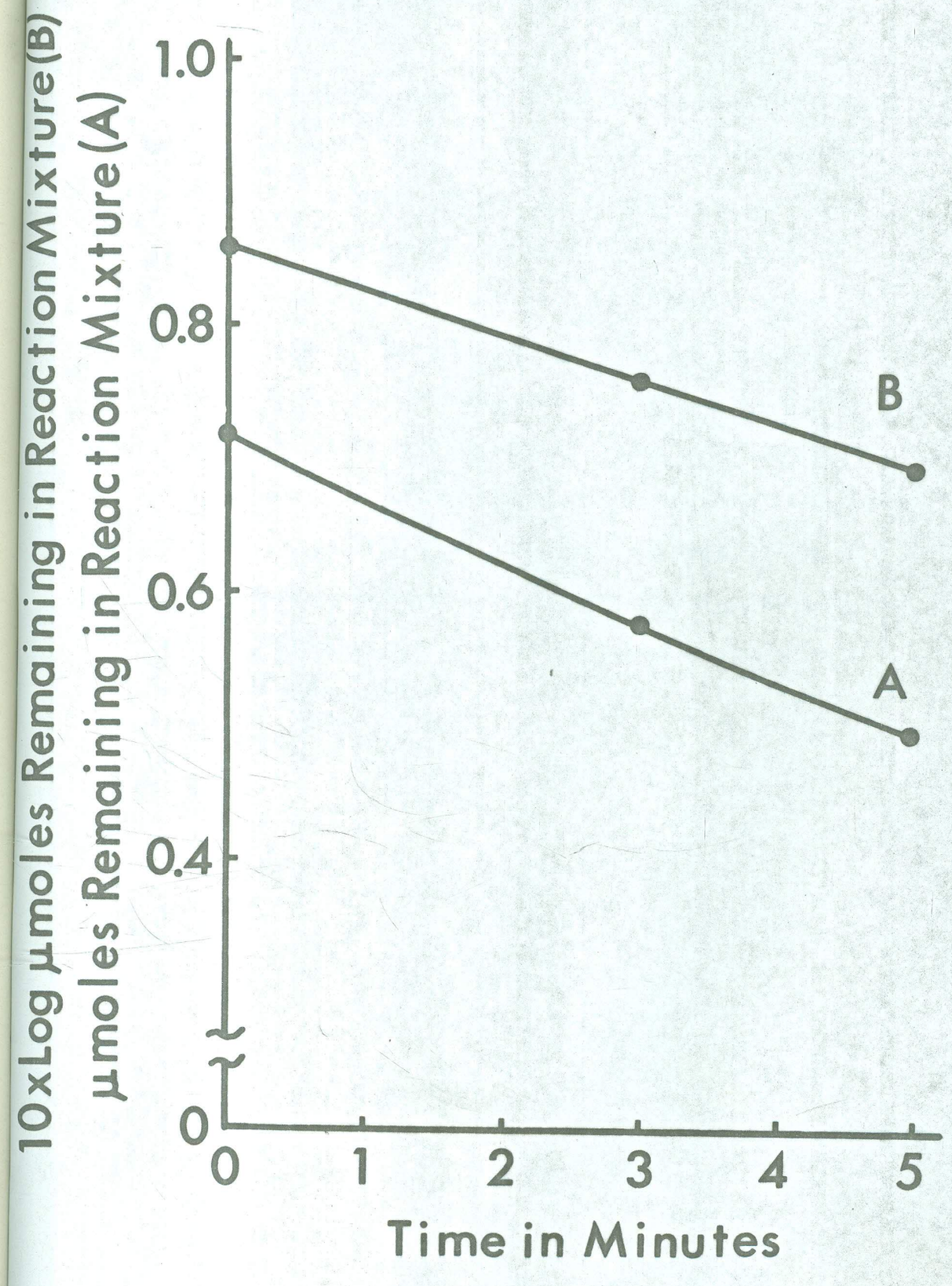

10xLog µmoles Remaining in Reaction Mixture(B)
µmoles Remaining in Reaction Mixture(A)
1.0
0.8
0.6
0.4
0
B
A
0
1
2
3
4
5
Time in Minutes

FIGURE 5

Kinetics of Maleylacetoacetate Isomerase.

Progress curves show the change of the concentration, linearly (A) and logarithmically (B), of maleylacetoacetate as a function of time. Enzyme was assayed by a fixed-time spectrophotometric method in Beckman B Spectrophotometer at 330 mμ. Reaction mixture consisted of 50 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.75, 2.5 μmoles of glutathione, 0.1 ml liver supernatant fraction, an amount of neutralized substrate preparation which would have an initial absorbance in the more sensitive range of the spectrophotometer (usually 1.0 ml) and water to a total volume of 3.0 ml. Control mixtures were made up similarly but with no substrate. Samples were assayed for maleylacetoacetate at zero time, 3 minutes and 5 minutes at 37°.

fumarylacetoacetate, was prepared by acid isomerization of maleylacetoacetate. The enzyme assay was carried out in the presence of N-ethylmaleimide to inhibit the isomerase (102), since significant amounts of maleylacetoacetate were present in the substrate as prepared.

The neutralized substrate was added to the reaction mixture containing 100 μmoles phosphate buffer, pH 7.5, 3.0 μmoles of N-ethylmaleimide, 0.1 ml of a 1:4 diluted liver supernatant fraction and water to a total volume of 3.0 ml. The blank was made similarly but with no substrate. The procedure of obtaining the zero-time, 3-minute and 5-minute samples, and the assay of maleylacetoacetate and fumarylacetoacetate were similar to those described for the isomerase.

The enzyme follows zero order kinetics, (Figure 6), in agreement with the finding by Edwards and Knox (104).

Figure 6

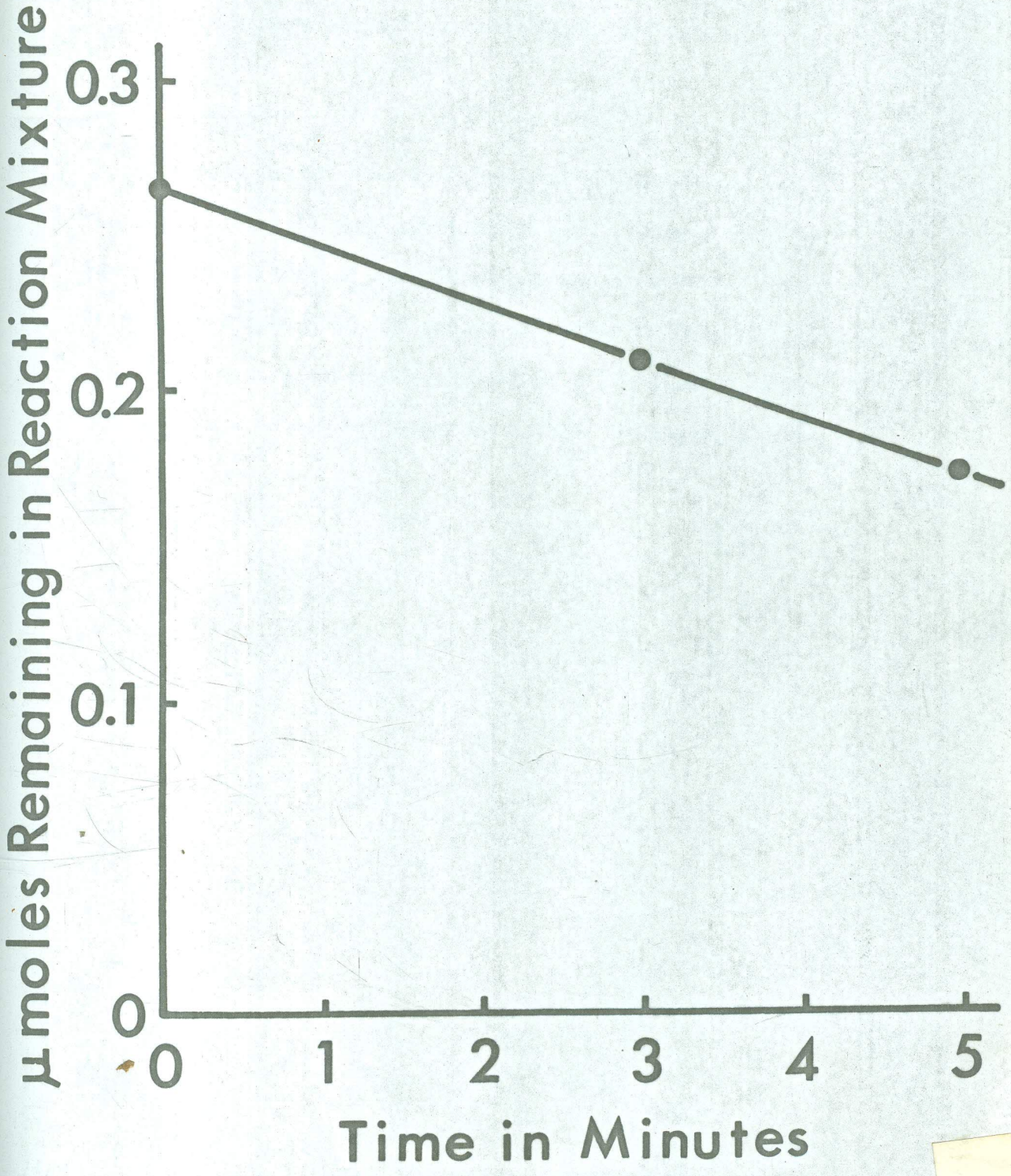

μ moles Remaining in Reaction Mixture
0.3
0.2
0.1
0
0
1
2
3
4
5
Time in Minutes

FIGURE 6

Kinetics of Fumarylacetoacetate Hydrolase.

Progress curve shows the change of concentration of fumarylacetoacetate as a function of time. Enzyme assay was similar to that of maleylacetoacetate isomerase. Reaction mixture contained 100 μmoles phosphate buffer, pH 7.5, 3.0 μmoles N-ethylmaleimide, 0.1 ml of a 1:4 diluted liver supernatant fraction and water to a total volume of 3.0 ml.

RESULTS

The levels of the five enzymes during the first day after birth and in the adult rat are given in Table 4 and illustrated in Figure 7. A variety of developmental patterns can be seen.

Tyrosine Transaminase:

The changes in the activity of the liver tyrosine transaminase of the young rat are very pronounced. The activity of the enzyme at birth is low, about 22% of that of the adult livers. There is little change in the enzyme activity in the first 1 1/2 hours, but it increases rapidly thereafter, attaining a maximum at 12 hours after birth. The enzyme activity at the peak is a little over double the observed activity of the adult rat. Then the activity decreases, so that by 24 hours it has reached a value slightly below that observed in the adult rat. These results are in agreement with those of Sereni, Kenney and Kretchmer (72).

p-Hydroxyphenylpyruvate Hydroxylase:

The activity of liver p-hydroxyphenylpyruvate hydroxylase in the young rat is about eight times higher than that of the

TABLE 4 (continued)

Litter Number	No. of Rats in Litter	Avg. Rat Wt. in Gm.	Avg. Liver Wt. in Gm.	Age (Hours)	Protein in Liver Supernat. mg/ml	Specific Activity in mU/mg except for MAA isomerase, which is in $mK_{(1st\ order)}$/mg				
						TAA	HPPA-HY	HGA-OX	MAA-IS	FAA-ase
8	17	5.47	0.31	12	14.9	22.4	22.4	13.8	31.4	81.9
9	13	5.87	0.29	12	16.0	19.9	25.7	14.0	30.2	80.4
				Mean	15.4	21.1	24.1	13.9	30.8	81.2
10	10	6.16	0.31	24	18.1	7.21	19.8	12.8	35.2	70.0
11	15	5.85	0.29	24	18.6	7.06	20.6	13.4	32.9	65.5
				Mean	18.4	7.13	20.2	13.1	34.1	67.7

Adult Rats

Weight of Rat in Gm.	Weight of Liver in Gm.	Sex						
302	11.5	Female	22.2	10.7	20.9	17.3	24.8	49.6
200	13.8	Male	18.3	8.80	27.4	16.6	29.4	41.9
120	12.3	Male	18.8	7.96	24.6	15.0	25.1	35.9
110	10.8	Male	21.3	8.23	23.2	15.8	26.3	37.2
		Mean	20.1	8.91	24.0	16.2	26.4	41.2

TABLE 4

Development of Tyrosine-Metabolizing Enzymes During the First Day after Birth of the Rat

Litter Number	No. of Rats in Litter	Avg. Rat Wt. in Gm.	Avg. Liver Wt. in Gm.	Age (Hours)	Protein in Liver Supernat. mg/ml	Specific Activity in mU/mg except for MAA isomerase, which is in $mK_{(1st\ order)}$/mg				
						TTA	HPPA-HY	HGA-OX	MAA-IS	FAA-ase
1	13	4.54	0.41	At Birth	9.00	2.67	22.3	22.7	25.0	75.9
7	12	5.67	0.41	At Birth	9.29	2.86	25.2	23.1	27.5	68.5
				Mean	9.14	2.76	23.7	22.9	26.3	72.2
3	12	4.80	0.36	1 1/2	9.45	2.02	23.1	12.8	28.9	109
14	13	5.20	0.34	1 1/2	9.81	3.36	26.0	17.3	27.8	105
				Mean	9.63	2.69	24.6	15.0	28.3	107
4	7	6.60	0.30	3	10.8	10.6	31.7	19.5	21.7	102
13	12	5.71	0.37	3	10.8	8.02	31.9	18.7	21.2	105
				Mean	10.8	9.28	31.8	19.1	21.5	104
6	10	5.73	0.36	6	12.5	15.6	30.0	15.7	30.1	83.5
12	10	5.45	0.34	6	12.3	13.7	25.3	16.6	32.9	83.7
				Mean	12.4	14.7	27.7	16.2	31.5	83.6

Figure 7

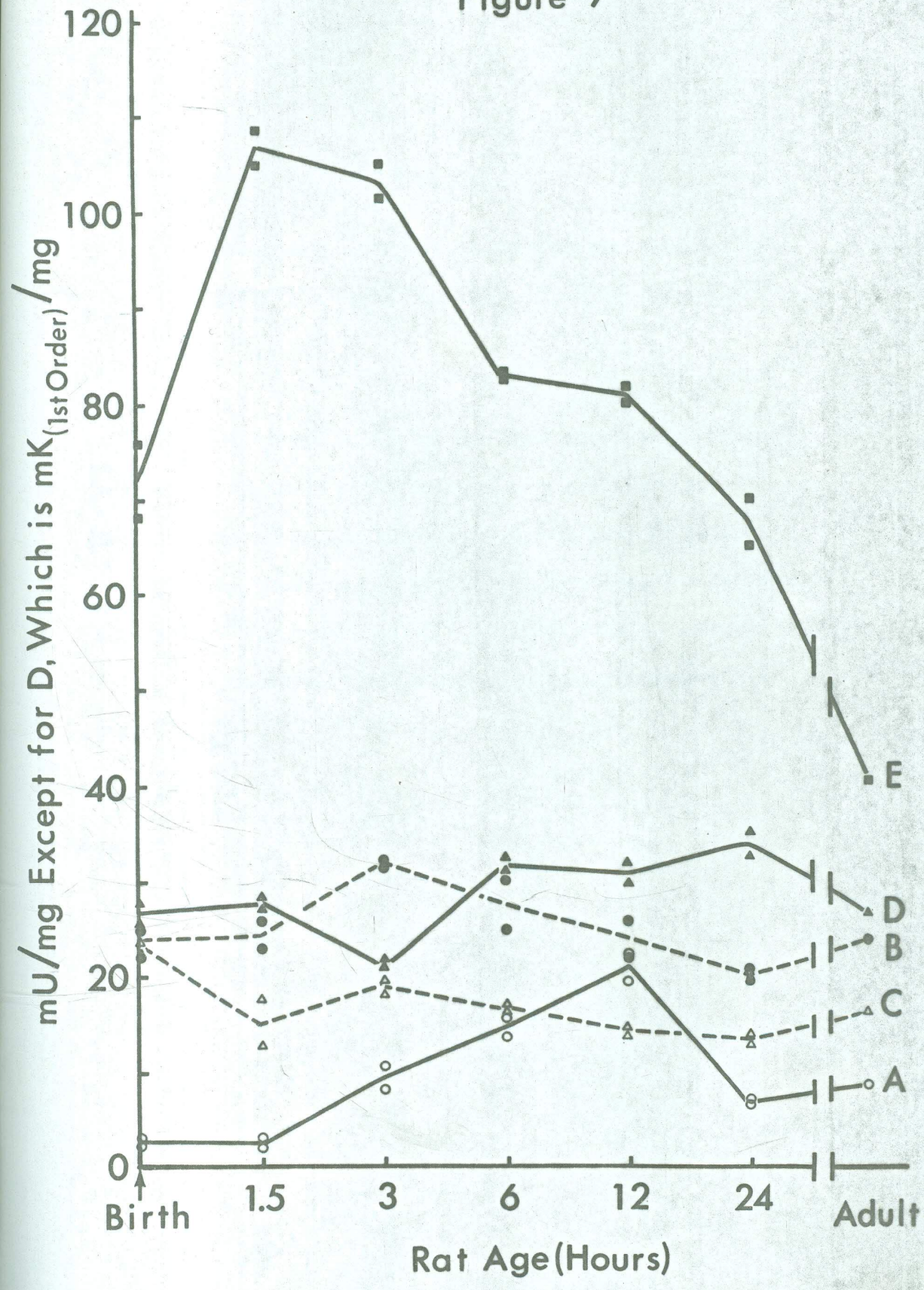

120
100
80
60
40
20
0
mU/mg Except for D, Which is mK(1st Order)/mg
E
D
B
C
A
Birth
1.5
3
6
12
24
Adult
Rat Age (Hours)

FIGURE 7

Development of Tyrosine-Metabolizing Enzymes During the First Day after Birth of the Rat.

Graphical presentation of the data reported in Table 4. Each upper and lower point represents one litter (pooled). Lines are drawn through the means of each pair of points. Abbreviations used: A = tyrosine transaminase; B = p-hydroxyphenylpyruvate hydroxylase; C = homogentisate oxygenase; D = maleylacetoacetate isomerase; E = fumarylacetoacetate hydrolase.

transaminase at birth and equal to that of the hydroxylase in the adult. Like the transaminase, little change is observed in the first 1 1/2 hours, but subsequently it increases rapidly, reaching a peak at 3 hours. Thereafter, the activity of the hydroxylase decreases gradually, reaching, at 12 hours after birth, the value observed at birth. At 24 hours its activity has decreased to a value a little below that of the adult rat.

Homogentisate Oxygenase:

At birth the activity of rat liver homogentisate oxygenase is very close to that of the hydroxylase and higher than the adult value. However, its activity decreases in the first 1 1/2 hours, followed by an increase, reaching a peak at 3 hours (but the activity remains lower than that at birth). A gradual decrease in the activity of this enzyme follows, reaching, at 24 hours, a value of half the activity observed at birth. At 24 hours the enzyme activity is slightly lower than the adult value. After diverging at birth, this and the preceding enzyme appear to follow parallel patterns.

Maleylacetoacetate Isomerase:

The changes in the activity of liver maleylacetoacetate isomerase appear to be less distinctive than what is observed for the other enzymes. Its activity at birth is quite close to that of the adult and very little change in enzyme activity

is observed in the first 1 1/2 hours. Thereafter, its activity drops and rises a few times, and by 24 hours its activity is somewhat higher than that observed in the adult.

Since isomerase activity is reported in terms of the first-order reaction rate constant rather than in standard units, strict comparison with activities of the other enzymes is not possible. However, for purposes of approximation, isomerase activities have been calculated also by the crude assumption that the initial phase of the logarithmic progress-curve of the reaction approximates a straight line (therefore, zero-order). On this basis, isomerase activity more or less follows the course plotted in Figure 7, but displaced somewhat lower, to the areas occupied by the hydroxylase and oxygenase.

Fumarylacetoacetate Hydrolase:

The developmental pattern of this enzyme appears to be quite different from all the other enzymes in this pathway. To begin with, its activity is much higher at birth than the rest; being 2 to 3 times higher than the hydroxylase and oxygenase. There is a striking increase in the activity in the first 1 1/2 hours, attaining a level which is about 2 1/2 times that of the adult. It then decreases steadily and by 24 hours the activity is back to what it was at birth, but still much higher than the enzyme activity observed in the adult rat.

DISCUSSION

The overall metabolic sequence and the enzymes responsible for catalyzing the oxidative degradation of tyrosine to acetoacetate and fumarate are quite well known. The newer problems open for study, therefore, are those of regulation. Since the tyrosine pathway occurs with no side-paths of any importance, it seemed possible that all the enzymes involved in this route are coordinated in their regulation. Two areas are available for investigation: (1) control in the adult, and (2) developmental control in the young. This project covers the second aspect.

The investigation of enzyme development adds a dimension to the study of biochemical differentiation. The central problem is how all cells of one organism possessing the same genome acquire very different functions in the mature animal. The regulation of enzyme activity poses some acute questions in systems in which both enzymes and regulators are also developing. The role of enzymes in the physiology of adult organs must be extended to reach the difficult problems, peculiar to the embryo, of induction and differentiation. At the level of gene action, investigations into enzyme development hold particular promise,

for the appearance or disappearance of an enzyme at a given site or organ may provide clues to the differential gene activities that must be basic to the understanding of problems of regulation. Unknown timing devices must turn on some genes and turn off others at the appropriate time. Other controls may operate at a non-genomic level, for example, at the ribosomal site.

Although the nature of the timing mechanism is unknown, the first step in such investigations is to establish the facts, that is, the resultant developmental patterns.

For most of this tyrosine pathway, the patterns have not been described in the literature. However, it was known that the transaminase underwent unusual fluctuations in the first 24 hours, and therefore it seemed of interest to investigate all of the enzymes during this time period.

One of the first problems faced in designing this project was that of deciding how to measure the metabolic reaction rates. Several approaches can be considered:

(1) In some way, one could assay the reactions in vivo, or use in vitro conditions which simulate those in the animal, to get an approximation of the actual status in vivo.

(2) One could assay the enzymes under optimum conditions in vitro. The activities will be maximal, and may well be faster than the reactions which can take place in vivo. However, they can serve as an approximation of how much apoenzyme is available or has been synthesized by a given age. They give an upper limit for conclusions as to in vivo rates.

The first approach is not feasible at present. The intracellular conditions for the tyrosine pathway, such as substrate concentration, cofactor concentration, pH, etc., are not known. Conditions for whole animal experiments (isotope labelling, etc.) are also not worked out as yet. Therefore, we used the second approach.

A second problem in designing this project was that of deciding how to express the results. Different methods of reporting enzyme measurements have been followed by research workers in this field. Enzyme units have been expressed per (a) whole organ or whole animal, or (b) unit weight of wet tissue, dry tissue, DNA-phosphorus (that is, per cell) or protein.

Methods (a) have the advantage of emphasizing the total metabolic capability available to the animal. However, this may not mean much when enzymes are assayed under unphysiological (that is, "optimum") conditions in vitro. All of the (b) methods have been used at one time or another; each has some advantages and disadvantages. We are using protein of the supernatant fraction as our basis, which relates the development of enzyme activities to the synthesis of total cytoplasmic protein, and indicates whether the former are developing faster than, at the same rate as, or slower than the latter. Hence differential rates of synthesis of specialized proteins will be brought to light.

An analysis of variance was performed on the experimental results, using the method described in Winer for repeated measurements (i.e., different enzymes) with the same subjects (105). With all five enzymes, there was, as expected, a highly significant interaction between enzymes and time. That is, there was significant departure from parallelism and, contrary to our original hopes, the enzymes did not appear to show coordinated patterns of development in the first 24 hours.

Since the hydrolase was obviously quite different from the other four, the analysis was repeated for the other four and again there was significant interaction between enzymes and time.

Only in the case of hydroxylase and oxygenase were the data consistent with the hypothesis of parallelism and this was true only when the analysis was restricted to 1 1/2 hours and later. These two then act as though they were coordinated and may be part of a "functional genic unit" (106). It may be fruitful to look for coordination in the control of these two enzymes in the adult.

One possible relationship among the enzymes can be seen in Figure 7, although it may be no more than a coincidence. With the exception of the isomerase, each enzyme shows a peak of activity at some point in the 24 hours, and these peaks appear to be timed in the reverse of the metabolic sequence: tyrosine transaminase at 12 hours, p-hydroxyphenylpyruvate hydroxylase and homogentisate oxygenase at 3 hours, and fumarylacetoacetate hydrolase at 1 1/2 hours. Is there a physiological mechanism which ensures that later reactions of a pathway are ready before earlier reactions are activated, and, therefore,

avoids any accumulation of intermediate products? Further work is required.

In terms of general levels on the activity scale, the first reaction of the metabolic sequence, the transaminase, usually has the lowest activity (except at the 12-hour period), and the last reaction, the hydrolase, has the highest activity, with the remaining three enzymes in between. The transaminase, although a reversible reaction, would be expected to be rate-limiting (again, with the possible exception of the 12-hour period, where the oxygenase may be rate-limiting), and hence would be the ideal target for regulatory mechanisms, as indeed is borne out by the literature. (It should be noted that we assay the hydroxylase in its fully activated form; if, in vivo, it is less than fully activated, it may well be rate-limiting along with the oxygenase at the 12-hour period).

We have no firm explanation for the very high activity of the hydrolase. Although later reactions in a metabolic sequence must be active enough to handle metabolites formed by earlier reactions in the sequence, the activity of the hydrolase seems to go far beyond this need. There is a possibility that the hydrolase is associated with the lysosomes and released due to the rupture of these particles during homogenization, making it impossible to distinguish between "free" and "bound" hydrolase. This possibility will have to be investigated in future experiments. Secondly, the substrate, fumarylacetoacetate, may be harmful to the cell in some way, requiring its removal immediately as it is formed. Finally, the enzyme may

have physiological functions other than the hydrolysis of this one substrate. It is important to note the probable identity of this enzyme with others found in the liver which hydrolyze certain synthetic substrates (91, 106, 107). Further work is required to see if the hydrolase has physiological substrates other than fumarylacetoacetate.

SUMMARY AND CONCLUSIONS

1. The literature was reviewed on the regulation of tyrosine metabolism in both the adult and developing rat.
2. In some cases, alterations were introduced in the general procedures for the assay of the enzymes involved in the tyrosine pathway, in order to suit our conditions.
3. Improved methods were established for the preparation of the two substrates not available commercially.
4. Activities of the five enzymes of tyrosine catabolism were assayed in the newborn rat at intervals from birth to 24 hours.
5. The activity of liver tyrosine transaminase was very low at birth, but it increased rapidly thereafter, reaching a peak of about twice the adult level at 12 hours. Subsequently it decreased, reaching a value slightly below that observed in the adult rat at 24 hours.

6. p-Hydroxyphenylpyruvate hydroxylase activity was present in the young rat at birth and equal to that of the hydroxylase activity in the adult. After the first 1 1/2 hours, the activity of the hydroxylase increased rapidly, reaching a peak at 3 hours, followed by a gradual decrease, reaching, at 12 hours after birth, the value observed at birth.
7. Similarly, homogentisate oxygenase was present at birth, followed by a decrease in the first 1 1/2 hours, after which this and the preceding enzyme appeared to follow parallel patterns.
8. Maleylacetoacetate isomerase was present at birth, but the developmental changes in the first 24 hours after birth appeared to be less distinctive than what was observed for the other enzymes.
9. The activity of fumarylacetoacetate hydrolase was much higher at birth than the rest of the enzymes involved in this pathway. It increased dramatically in the first 1 1/2 hours, then decreased steadily, reaching, by 24 hours, an activity which was still much higher than that observed in the adult rat.
10. After discussion of the rationale and the problems involved in designing this project, various explanations and interpretations of the results were offered, and areas for further research were indicated.

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