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REGULATION OF THE SYNTHESIS AND DEGRADATION OF KYNURENINE

by
Brenda Dale Manning

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1972

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ABBREVIATIONS

5-hydroxytryptamine (serotonin)	5OHT
tryptophan oxygenase (EC 1.13.1.12)	TO
kynurenine aminotransferase (EC 2.6.1.7)	KT
kynurenine hydrolase (EC 3.7.1.3)	KH
kynurenine	kyn
3-hydroxykynurenine	3OHkyn
pyridoxal-5-phosphate	PLP
kynurenic acid	KA
xanthurenic acid	XA
vitamin B_6	B_6
3-hydroxyanthranilic acid	3OHAA
nicotinamide adenine dinucleotide	NAD
nicotinamide adenine dinucleotide phosphate	NADP
nicotinamide mononucleotide	NMN
adrenocorticotropic hormone	ACTH
17 β-estradiol	ES
estradiol benzoate	EB
cortisol-21-phosphate	CP
testosterone propionate	TP
potassium phosphate	P_i
sodium pyrophosphate	PP_i
α-ketoglutarate	αkg
trichloroacetic acid	TCA
imidazole	Im
diethylstilbestrol disulfate	SDS

α-ketoadipate		αka
Michaelis constant		K_m
$\frac{\text{activity assayed in the absence of added PLP}}{\text{activity assayed in the presence of added PLP}}$	x 100	% saturation

INTRODUCTION

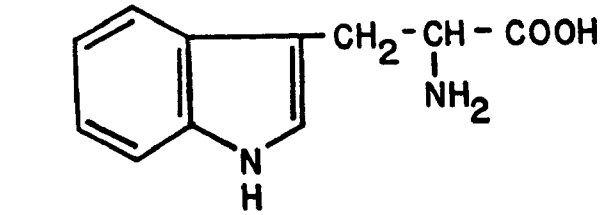
Tryptophan and agents that alter tryptophan metabolism are widely used with human subjects for medicinal, experimental, and diagnostic purposes. The potential benefits or undesirable effects of intervention in tryptophan metabolism would be more predictable if the regulation of tryptophan metabolism in mammals were more completely understood. This thesis research was undertaken in order to develop a better understanding of the regulation of tryptophan metabolism.

Recently a great deal of effort and interest has been directed toward the study of the minor pathway by which tryptophan is converted to 5-hydroxytryptamine (serotonin, 5OHT) in the brain (Fig. I). It is now known that this pathway does not function independently of the major degradative pathways which regulate the circulating levels of tryptophan and its metabolites (Fig. II). Therefore, knowledge of the regulation of these systemic pathways is applicable to those aspects of neural function that are influenced by the level of tryptophan metabolites in the brain.

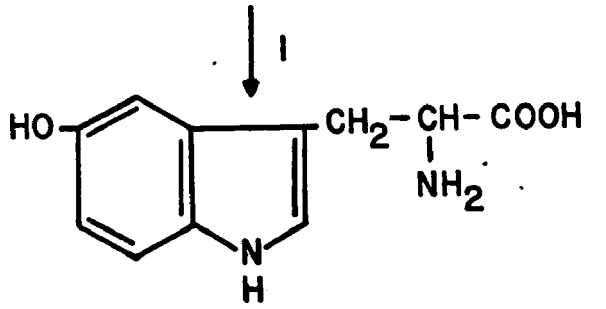
Most of the earlier studies on the regulation of tryptophan metabolism were concerned with the role of corticosteroids and tryptophan in regulating the level of tryptophan oxygenase in liver. Little was known about the control of other enzymes of the degradative pathways. The major focus of our research was to study the regulation of certain other enzymes of tryptophan catabolism by steroid hormones. The enzymes chosen for this study were tryptophan

Figure I

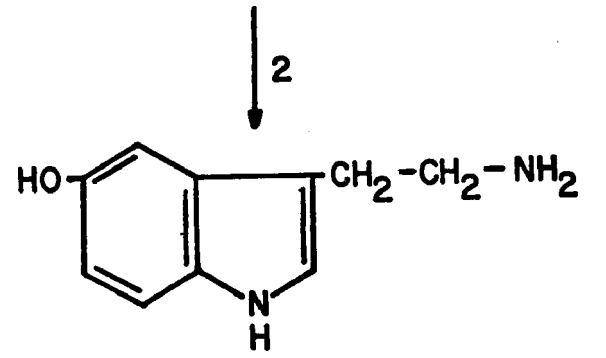
5-hydroxytryptamine Synthesis from Tryptophan



Tryptophan



5-hydroxytryptophan



5-hydroxytryptamine (serotonin)

(1) tryptophan-5-monooxygenase

(2) aromatic L-amino acid decarboxylase

Legend for Figure II

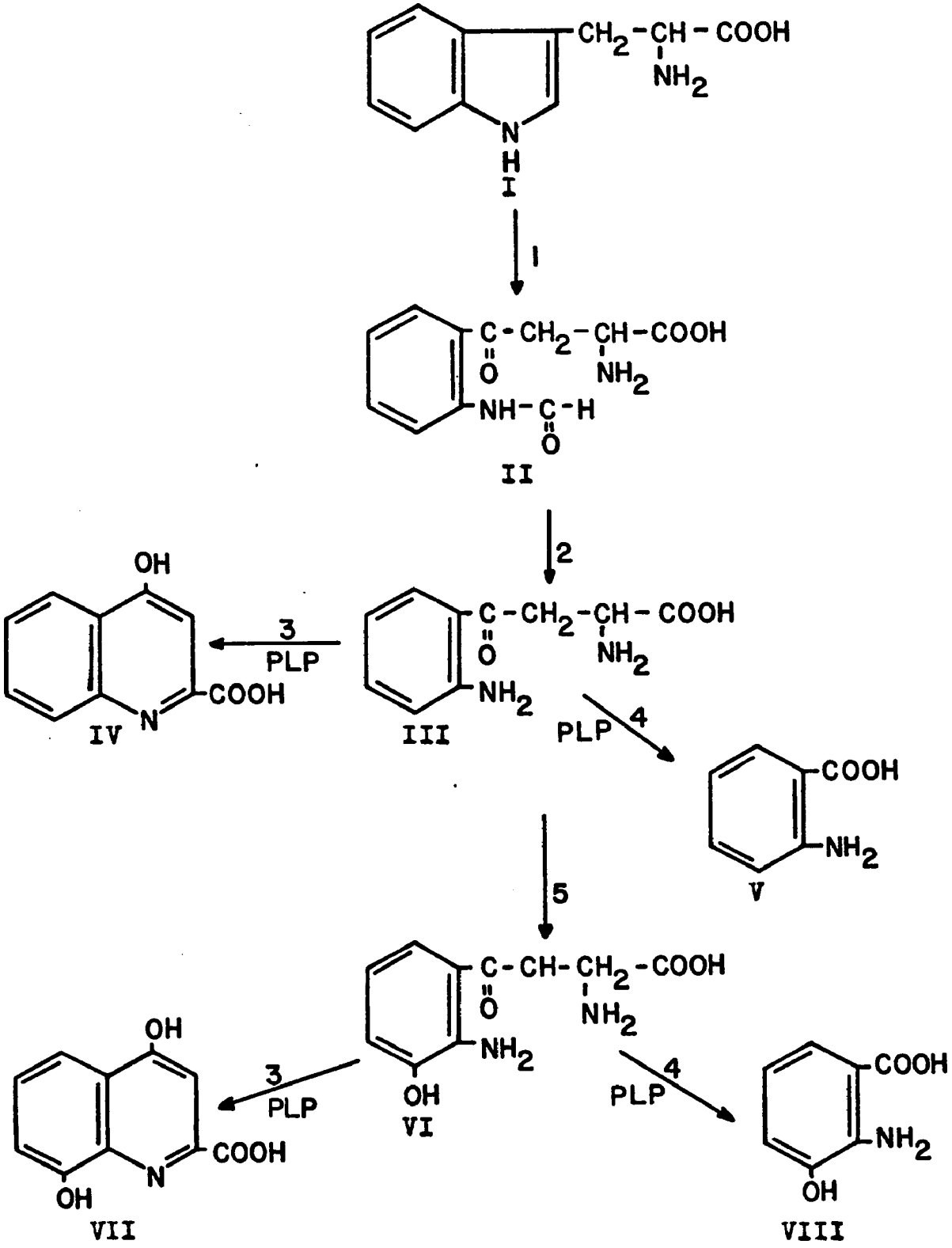
I. Tryptophan
II. Formylkynurenine
III. Kynurenine
IV. Kynurenic acid
V. Anthranilic acid
VI. 3-hydroxykynurenine
VII. Xanthurenic acid
VIII. 3-hydroxyanthranilic acid

(1) tryptophan oxygenase
(2) kynurenine formylase
(3) kynurenine aminotransferase
(4) kynurenine hydrolase
(5) kynurenine hydroxylase

Figure II

The Degradative Pathway of Tryptophan

via Kynurenine Intermediates



oxygenase (TO), kynurenine aminotransferase (KT), and kynurenine hydrolase (KH).

These three enzymes were selected for several reasons. The regulatory control of TO has been well established for the corticosteroids but not for other steroid hormones. KT and KH are branch-point enzymes which direct kynurenine (kyn) and 3-hydroxy kynurenine (3OHkyn) into alternate degradative pathways. Mechanisms must exist to regulate the extent to which each pathway is used.

Both KT and KH require pyridoxal-5-phosphate (PLP) as a cofactor and have been reported to decline in level during a pyridoxine deficiency state. The possibility existed that variations in enzyme levels could be mediated by the availability of this coenzyme.

These two enzymes also exhibit different organ and intracellular locations (1, 10). In liver homogenates the KH is found in the soluble fraction and the KT in the mitochondrial fraction. Therefore, the inner membrane of the mitochondria is a possible regulatory factor for it is well known to exhibit selective permeability and to function in the regulation of other aspects of metabolism. There is very little KH in the kidney but KT occurs in both the soluble and mitochondrial fractions of kidney homogenates. Here again the mitochondrial membrane may be a regulatory factor. The different organ locations may also play a role in regulation for many hormonal effects and other

regulatory factors are known to be organ specific.

KT and KH have both been shown to exhibit sex differentiation in adult rats and there is some question as to whether such differentiation occurs with TO. Studies of these enzyme levels in male and female rats could give a better picture of the sex differentiation of tryptophan metabolism in rats.

Finally, previous studies have indicated that during pregnancy or estrogen administration there is a much greater than normal accumulation of kynurenine (kyn), kynurenic acid (KA), and xanthurenic acid (XA). These alterations could be the result of increased TO activity or decreased degradation of kyn via KH. Studies of hormonal alterations of these enzyme levels could delineate these possibilities.

We decided to study variations in these enzyme activities in both liver and kidney and both soluble and mitochondrial fractions. Activities were measured at various times of day, at various stages of growth, and with various hormonal alterations. We chose to study the effects of sustained, rather than short term, hormonal alterations resulting from oral or parenteral treatment of one week to several weeks. The sustained treatment was assumed to be more nearly representative of conditions existing during sexual differentiation and long-term administration of estrogens and contraceptive steroids. The use of oral administration of steroids is not

conventional in animal studies but does provide a somewhat analagous condition to that of the excretion studies in which oral administration of estrogens was used.

The results and discussion of this thesis research will be reported in two parts. Part I will deal with the whole animal experiments. In order to obtain reliable information about the enzyme activities we had to study the *in vitro* characteristics of the enzymes. These studies were also done to determine if we could detect any properties of the enzymes that might be of regulatory significance. Part II presents this *in vitro* data.

HISTORICAL BACKGROUND

A. Pathways of Tryptophan Metabolism

A general review of tryptophan metabolism has been compiled by Meister (2). The degradative pathways in mammals will be briefly summarized here with specific reference to rats and man.

The major pathway of tryptophan degradation in mammals involves oxidation via kyn and related intermediates to acetyl-CoA, CO_2, and urea. The metabolic sequences were established by isolating tryptophan metabolites in feeding and labeling experiments (3-8). The involvement of vitamin B_6 (B_6) in tryptophan metabolism was demonstrated by the observation that XA was greatly increased in the urine of B_6-deficient rats fed a dose of tryptophan. These early metabolite studies were soon followed by enzyme isolation and characterization studies (9-19). The established metabolic sequence is summarized in Fig. II and the enzymic requirements for B_6 coenzymes are indicated.

It was long known that tryptophan could replace nicotinic acid as a growth factor in rats (20) and that 3-hydroxyanthranilic acid (3OHAA) was an intermediate between tryptophan and NAD (21). The major quantitative degradation of kyn is now thought to be complete breakdown to CO_2, NH_3, and acetyl CoA. Not until recently however have these pathways been completely established (22-24). Fig. III summarizes both sequences.

Another pathway leads to the formation of 5OHT which

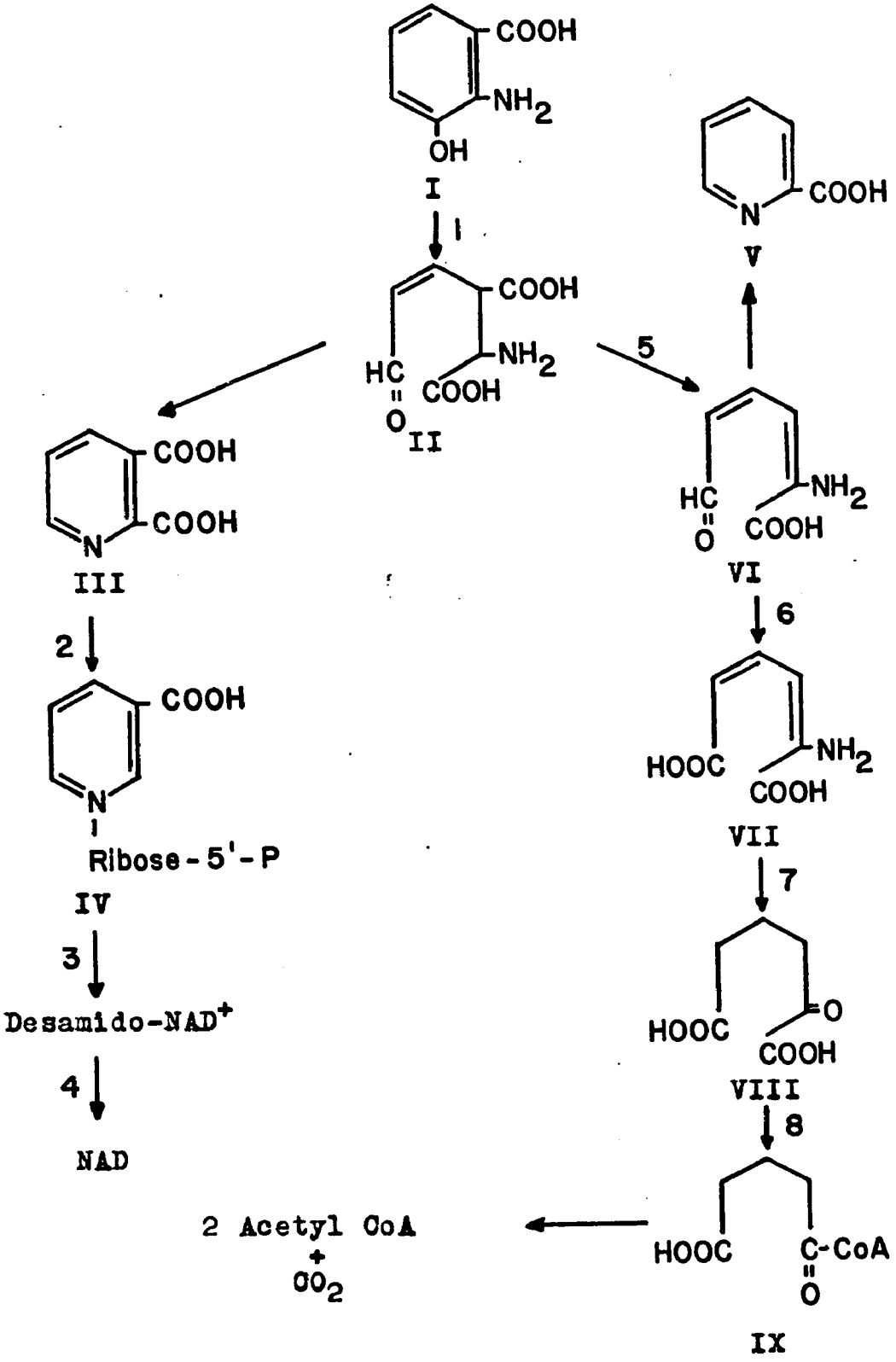
Legend for Figure III

I. 3-hydroxyanthranilic acid
II. α-amino-β-carboxymuconic-ε-semialdehyde
III. quinolinic acid
IV. nicotinic acid ribonucleotide
V. picolinic acid
VI. α-aminomuconic-ε-semialdehyde
VII. α-aminomuconic acid
VIII. α-ketoadipic acid
IX. glutaryl CoA

(1) 3-hydroxyanthranilic acid oxidase
(2) quinolinate phosphoribosyltransferase
(3) nicotinic acid ribonucleotide pyrophosphorylase
(4) NAD synthetase
(5) picolinic carboxylase
(6) α-aminomuconic-ε-semialdehyde dehydrogenase
(7) α-aminomuconic acid reductase
(8) α-ketoadipate (or α-ketoglutarate) dehydrogenase

Figure III

The Pathway of 3-hydroxyanthranilic Acid to NAD, Acetyl CoA, and CO_2



has been shown to possess potent pharmacological activities. Udenfriend et al. (25, 26) have established that 5-hydroxy tryptophan is an intermediate in 5OHT synthesis (Fig. I). The site of hydroxylation of tryptophan for brain 5OHT synthesis appears to be intracerebral in rats (27, 28) although systemic hydroxylation of tryptophan also occurs.

Since this thesis research is concerned mainly with three enzymes of the tryptophan pathway their reactions may be of interest. KH uses either kyn or 3OHkyn as substrate yielding alanine and anthranilic acid (AA) or 3OHAA respectively (Fig. IV) (29). KT also utilizes either kyn or 3OHkyn to yield KA and XA respectively (Fig. V). TO (Fig. II) is a dioxygenase requiring tryptophan and O_2 as substrates and heme as a coenzyme (12, 14, 15). One atom of atmospheric oxygen is incorporated into kyn and the other into the formic acid formed by the combined action of TO and kynurenine formylase (14).

B. Regulation of Tryptophan Metabolism

Little is actually known about the control and regulation of tryptophan metabolism. Several enzymes and intermediates have been studied extensively but only a vague understanding has resulted so far. This summary will cover non-hormonal regulation and then hormonal regulation.

Cho Chung and Pitot (30) carried out inhibition studies with a 1400-fold purified TO preparation. The following compounds were found to inhibit the enzyme:

Figure IV

Kynurenine Hydrolase Reaction

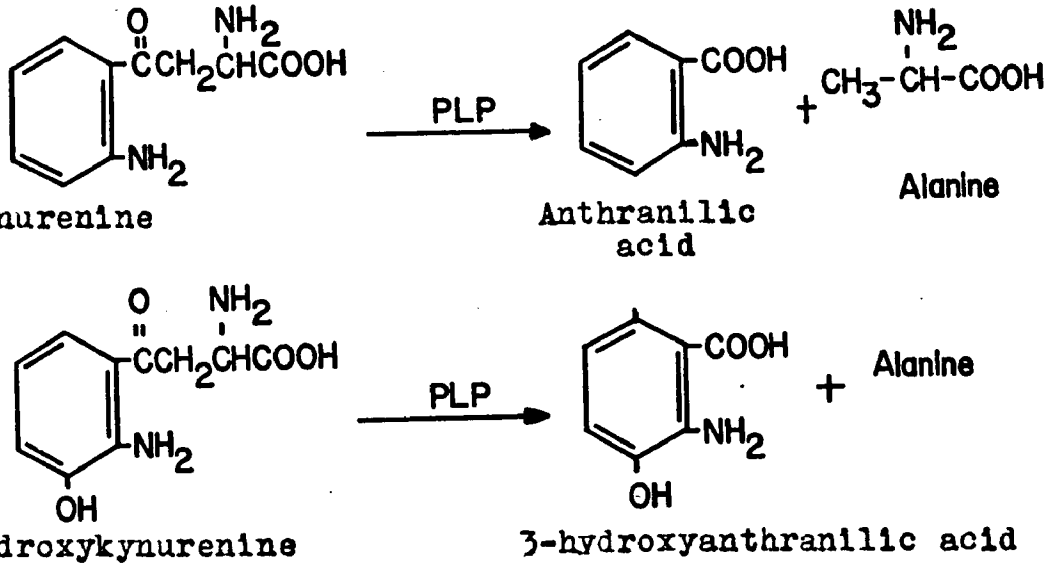
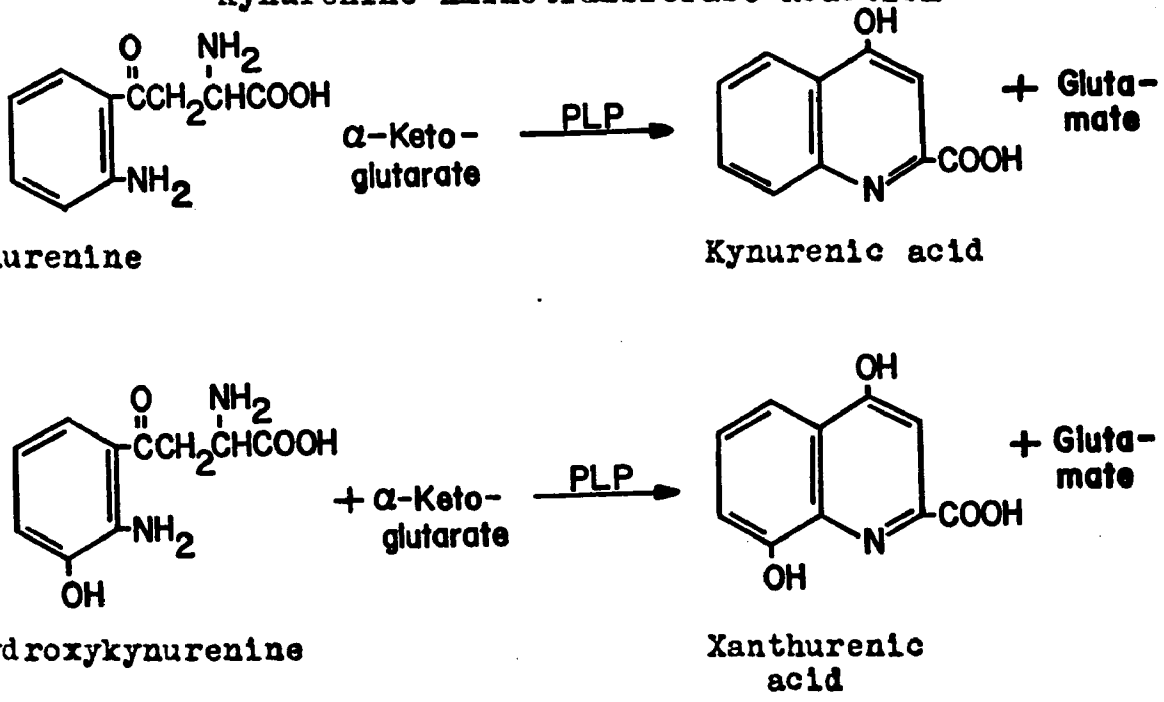


Figure V

Kynurenine Aminotransferase Reaction



NADPH > NADH > NADP > desamido-NAD > NMN > NAD > nicotinic acid They studied the kinetics of the NADPH inhibition and found that inhibition to be allosteric. Hill plots indicated that there were at least two binding sites for NADPH per enzyme molecule. Two fractions containing enzyme activity were obtained from a Sephadex G-200 column. The heavier, highly-active form was sensitive to NADPH inhibition whereas the lighter, relatively-inactive form was insensitive to NADPH inhibition. The authors stated that the inhibition data could most simply be explained by the NADPH causing a disaggregation of a TO polymeric form to an inactive monomeric form but further evidence is needed to substantiate this possibility.

It is now well established that injected tryptophan can increase TO activity by favoring the formation of holoenzyme from apoenzyme and hematin (31-33). Recently it has been found that tryptophan may also increase TO activity in vivo by an actinomycin-sensitive mechanism (34).

3OHAA was known to be a competitive inhibitor of TO and to affect mitochondrial metabolism and physiology (35-39). This metabolite inhibited <u>in vitro</u> the oxidation of α-ketoglutarate (αkg) and uncoupled phosphorylation from oxidation in mitochondrial preparations. 3OHAA also stimulated the adenosine triphosphatase and caused mitochondrial swelling.

B_6-deficiency has profound effects on tryptophan

metabolism (1, 60). As expected the deficiency leads to a lower degree of saturation of the B_6-requiring enzymes, KH and KT. The soluble fraction enzymes are affected to a greater extent than the mitochondrial enzymes. The kidney supernatant KT and liver KH apoenzyme levels also decrease. It has been reported that the KH activity is depleted more than the total KT activity. This observation led to the suggestion that the increase in excretion of the aminotransferase products characteristic of B_6-deficient animals given tryptophan is the result of diversion of metabolism from the major degradative pathway to the alternate one (1).

The most thoroughly studied aspect of hormonal regulation of tryptophan metabolism is the effect of glucocorticoids on TO activity. It has been well established that cortisone (40) and ACTH (41) induced TO activity and that adrenalectomy (40) lowered the activity in rats. The induction was shown to result from an increased rate of synthesis of the enzyme (42).

Circadian rhythms (43, 44) of metabolite levels and enzyme activity levels might have a profound effect on tryptophan metabolism. Although TO exhibits a circadian rhythm very similar to that of blood and liver tryptophan levels, there are some indications that the tryptophan rhythm does not generate the TO rhythm (45). The plasma levels of various hormones, which will be discussed below, also exhibit circadian rhythms (44).

Interest in the regulation of tryptophan metabolism was stimulated by findings that pregnancy and the ingestion of oral contraceptives caused abnormal excretion rates of some tryptophan metabolites in women who had been given a dose of tryptophan (46-52). The changes in urinary excretion rates were reminiscent of those found in the B_6-deficiency states already mentioned. A possible deficiency of pyridoxine under these conditions was suggested by the observation that abnormal excretion rates caused by pregnancy and contraceptive pills could be prevented by the administration of pyridoxine (47-52).

Aurrichio et al. reported that TO was elevated in pregnant rats (53) but estrone plus progesterone treatment of female rats did not produce an increase in TO activity (54). Braidman and Rose have shown that progesterone administration to rats decreased TO activity and that progesterone inhibits the TO activity in vitro (55). The inhibition appeared to be competitive. Injection of female rats with estradiol benzoate doubled the TO activity (56). Thus it appears that estrogens may increase TO activity, at least in females, whereas progesterone may decrease this activity. These results are only preliminary however.

Mason and Gullekson found that estrogen sulfates inhibited KT activity of rat kidney homogenates by interfering with PLP binding to the apoenzyme (57, 58). These sulfates also protected the apoenzyme from proteolytic digestion. The ability of these estrogen derivatives to

interfere with PLP binding has been proposed (59) as a possible basis for the similarities of the effects of estrogen treatments and dietary B_6-deficiency in increasing the excretion of tryptophan metabolites. The normalization of the estrogen-induced excretion pattern by administered B_6 was consistent with this interpretation.

Mason et al. have also compared the activities of KT in male and female rats. Mature males had higher activity levels of kidney KT than mature females for both the supernatant and mitochondrial fractions. Ovariectomy resulted in higher enzyme activities for females and diethylstilbestrol treatment lowered the male activities (60). The liver mitochondrial KT showed no sex differentiation with respect to this activity level. Ovariectomy and estrogen treatment were without effect. In this same study B_6-deficient male rats were compared with control males and females to determine if the B_6-deficiency resulted in enzyme levels similar to those of females. There was a strong depression of the kidney supernatant KT activity but little decrease in the kidney mitochondrial KT activity. They concluded that the estrogen effect on tryptophan metabolism could not be the result of depletion of PLP of the type occurring in B_6-deficiency although a more localized effect on PLP metabolism or distribution remains a possibility.

Similar comparisons were made for males and females with respect to the KH activity (61, 62). As with the

KT, males had higher KH activity levels than females. Ovariectomy resulted in increased female activity levels and estradiol benzoate (EB) treatment of males resulted in lowered activity levels.

Estrogens have been shown to increase the blood levels of corticosteroid-binding globulin (transcortin) and thereby increase the level of circulating corticosteroids (63). In view of the known effects of corticosteroids on TO it has been proposed (59) that the estrogen effects on tryptophan metabolism are due to the direct effects of the corticosteroids on the TO activity. Since there is also a greater plasma corticosteroid level in female than in male rats (64), it is also possible that the observed estrogen-dependent sex differences are mediated by the corticosteroid effects on TO levels. Some of our experiments were designed to test whether TO, KH, and KT activities varied together or independently.

METHODS AND MATERIALS

Animals - Albino rats were purchased from Spartan Farms, Plymouth, Michigan. They were maintained on Rockland Rat Diet (unless specified otherwise) ad libitum with a 12:12 hour light:dark schedule changing at 6:00.

Solid Synthetic Diet - A special diet was prepared for the animals given L-tryptophan orally and their controls. The diet contained per kg: vitamin-free casein (250 g), glucose (580 g), Alphacel (40 g), salt mixture (20 g), vitamin mixture ($-B_6$) (20 g), Pyridoxine HCl (20 mg), cod liver oil (20 g), and corn oil (70 g). L-tryptophan (10 g) was added per kg of diet for the tryptophan-fed animals.

Tryptophan Oxygenase Assay - The method of Seglen and Jervell (65) was used. The animals were decapitated and the livers were quickly removed and placed in ice. Liver tissue was homogenized in freshly-prepared 0.02 M P_i buffer, pH 7.0, containing 2.5 mM L-tryptophan and 2.0 µM methemoglobin to yield 1% or 2% homogenates. Four ml aliquots were incubated at 37° in a metabolic shaker for 20 and 40 minute periods in duplicate. Identical 4-ml aliquots were deproteinized immediately without incubation to serve as blanks. The reaction was stopped by the addition of 1 ml of 15% metaphosphoric acid. The suspensions were filtered and the filtrates neutralized with 1 N NaOH. The optical density of the neutralized filtrate was read at 365 mu to determine kyn formation. The amount of kyn produced during the period between 20 and 40 minutes of

incubation was used for rate measurements. That rate was most nearly linear following the initial lag period.

Tissue Fractionation Procedure - Animals were killed by a blow to the head and decapitation. The liver and kidneys were removed immediately and placed in ice. The kidneys and the whole liver were homogenized in 4 volumes of cold 0.25 M sucrose. In order to minimize redistribution of components the mitochondrial and supernatant fractions were separated as quickly as possible. The homogenates were immediately centrifuged at 30,000 g for 20 minutes. Supernatant fractions were decanted and stored in ice. The pellets were resuspended in the original volume of sucrose solution by homogenization and centrifuged at 12,000 g for 20 minutes. The supernatants were decanted and discarded. The residues were again resuspended by homogenization in the original volume of sucrose and centrifuged at ~ 280 g for 10 minutes. The supernatant fractions were decanted and stored in ice. They are referred to later as "kidney mitochondrial" and "liver mitochondrial" fractions.

Kynurenine Hydrolase Assay - The incubation mixture (1.2 ml) contained 0.05 M sodium pyrophosphate (PP_i) buffer, pH 8.0, or 0.05 M P_i buffer, pH 7.8, 1×10^{-4} M PLP, 1.54×10^{-3} M L-kyn sulfate, and 0.4 ml (80 mg wet weight of tissue) of liver supernatant fraction. The mixture was incubated for 30 minutes at 37° after which time the reaction was stopped by the addition of 5 ml of 5% trichloroacetic acid (TCA).

The mixture was centrifuged and 3 ml of the supernatant were mixed with 6 ml of 5% TCA. The amount of anthranilic acid in the TCA solution was determined by a modification (66) of the method of Bratton and Marshall (67) which destroys the diazotized derivative of the residual kyn.

Kynurenine Aminotransferase Assay - The incubation mixture (0.6 ml) contained 0.05 M P_i buffer, pH 6.4, 1×10^{-4} M PLP, 5×10^{-3} M αkg, 3×10^{-3} M L-kyn sulfate, and 0.1 ml (20 mg wet weight of tissue) of enzyme fraction. The mixture was incubated for 30 minutes at 37° after which time 10 ml of 1% boric acid in 95% ethanol were added for deproteinization. After centrifuging, the optical density was read at 333 mu and 365 mu. These values were used to calculate the amount of KA produced (68).

In later experiments the incubation mixture contained 0.1 M imidazole (Im) buffer, pH 6.5, instead of the P_i buffer. Mason found (unpublished experiments) that mitochondrial KT activity was lower in Im buffer than in P_i buffer and that adding P_i to the Im buffer increased the activity. $CaCl_2$ at 10^{-3} M was found to increase the mitochondrial activity even more. Ca^{++} appeared to be required for maximal rates of penetration of the keto acid substrate through the inner mitochondrial membrane. Supernatant activity was about the same in the two buffer systems and Ca^{++} was without effect. These data (Fig. VI) show that the kidney mitochondrial KT levels obtained with P_i buffer were underestimated. This discrepancy did not invalidate

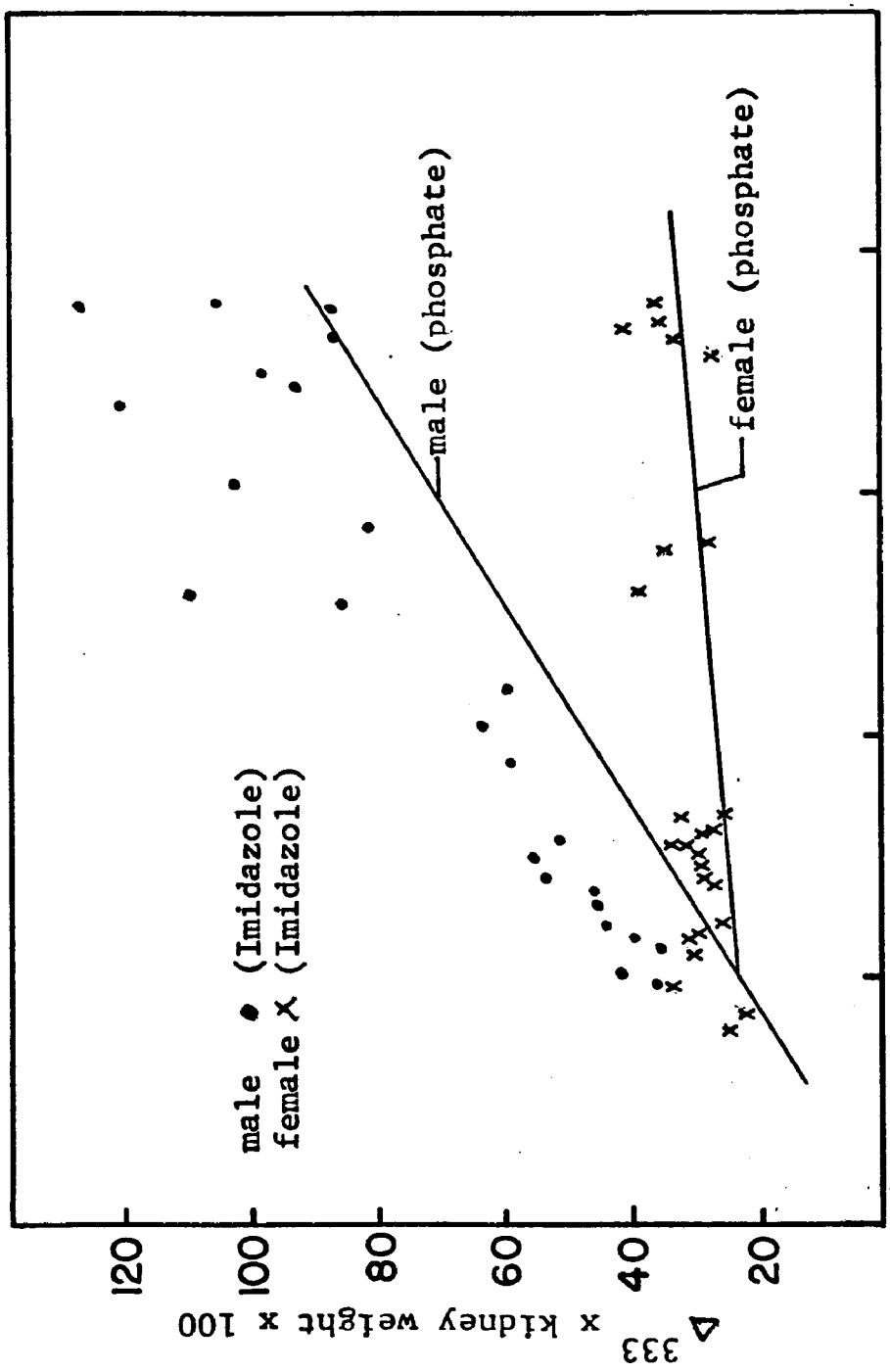
Legend for Figure VI

The fractions were prepared in 0.25 M sucrose as described in the Methods section. The incubation mixture (0.6 ml) contained 0.05 M P_i buffer, pH 6.4, or 0.1 M Im buffer, pH 6.5, 0.1 mM PLP, 5 mM α-kg, 3 mM $kynSO_4$, and 0.1 ml of enzyme fraction (equivalent to 20 mg wet weight of tissue). Incubation time was 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

All assays in Im buffer were done in the presence of 10 mM $CaCl_2$. The lines representing activity in phosphate buffer were derived from data obtained for a large number of animals of various body weights. The points are omitted in order to avoid confusion. The lines only represent the general trend of the data as determined by eye.

Figure VI

Comparison of Kidney Mitochondrial Kynurenine Aminotransferase Activity Assayed in Phosphate or Imidazole Buffer for Male and Female Rats



conclusions drawn from the earlier studies however. The activities of liver mitochondrial KT obtained in Ca^{++}-containing Im buffer were only slightly higher than those obtained with P_i buffer.

Materials

Diethylstilbestrol, estradiol benzoate, cortisol-21-phosphate (disodium salt), testosterone propionate pyridoxal-5-phosphate hydrochloride, L-kynurenine sulfate, α-ketoglutaric acid, crystalline bovine hemoglobin, and anthranilic acid were purchased from Sigma Chemical Company. Pyridoxine HCl, vitamin free casein, salt mixture (H.M.W.), vitamin diet fortification mixture (minus pyridoxine), and Alphacel (powdered cellulose) were purchased from Nutritional Biochemicals Corporation.

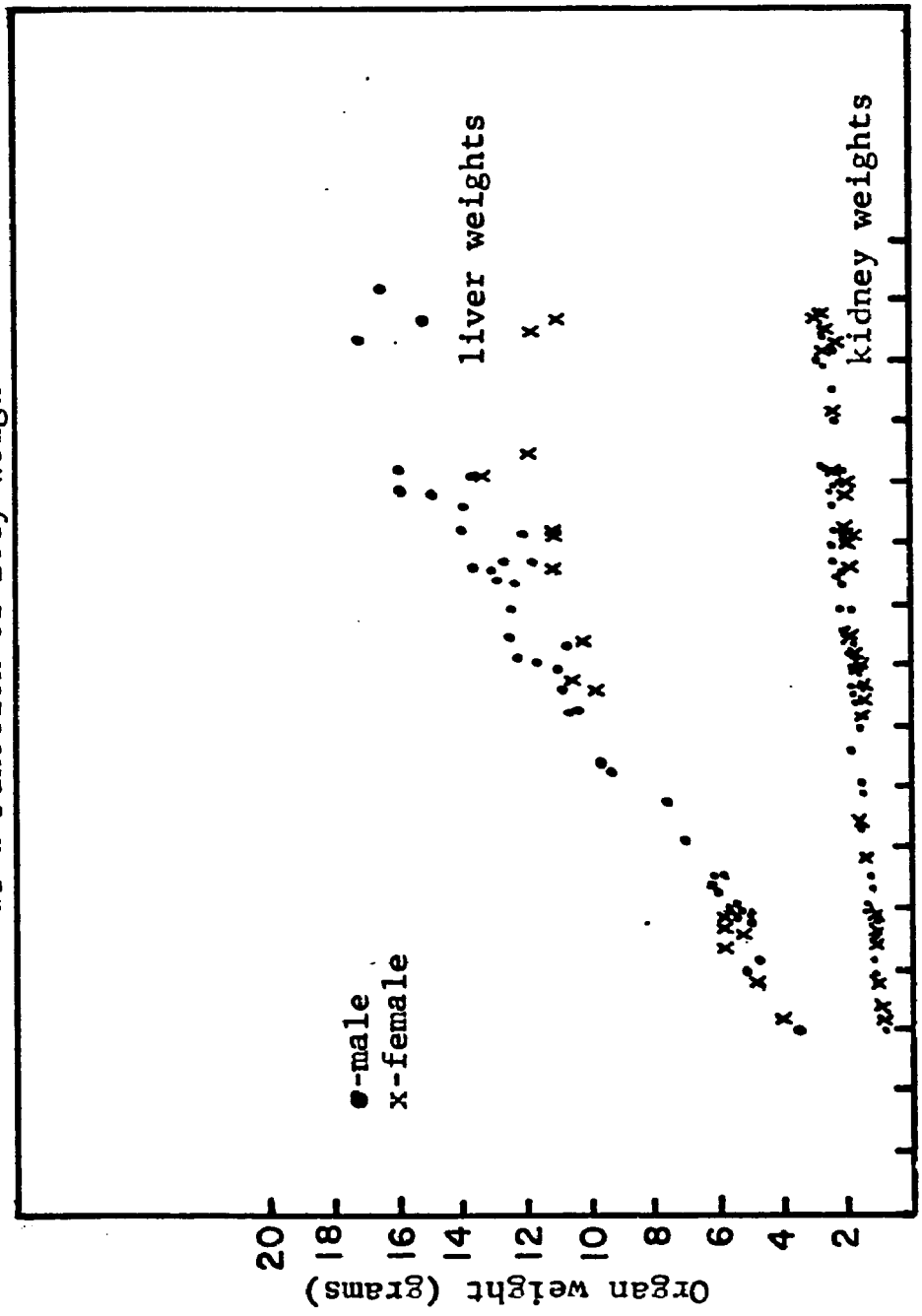
Methods of Reporting Data

Specific Activity - Specific activity is defined as μmoles of product made per hour per g wet weight of tissue.

Normalized Activity - This value was obtained by dividing the total organ activity (specific activity times organ weight) by the body weight and multiplying by 100. Mean normalized activities ± standard deviations are presented. Fig. VII presents the relationship between organ weight and body weight for male and female rats. This relationship is approximately linear for male rats, but departs from linearity with females livers at body weights above 200 g. Several of the hormonal treatments resulted in growth retardation, presenting us with the problem of

Figure VII

Comparison of Male and Female Liver and Kidney Weights as a Function of Body Weight



comparing animals of different body weights. The normalized activity takes this difference in body weights into consideration in comparing male and female values as a function of body weights. In drawing conclusions from results obtained in such experiments, both the specific activity and normalized activity must be considered together.

It should be noted also that sex differences and hormonally-induced differences in enzyme levels generally increase with increasing body weight (Fig. VI). The mean values, therefore, tend to understate the degree of difference in the heavier animals, if there is a substantial weight range. All data presented in this form have also been plotted in terms of total organ activity versus body weight. Such comparisons have not resulted in interpretations at variance with those presented and are, therefore, not presented.

Standard Deviation from the Arithmetic Mean - These values were obtained by using the formula

$$S.\ D. = \sqrt{\frac{\Sigma(x-x)^2}{N-1}}$$

$(x-x)^2$ = the squared deviation from the mean

N = number of values

Significance - P is the probability that the observed change occurred by chance as derived from the t test.

PART I

WHOLE ANIMAL STUDIES

RESULTS

Diurnal Variation of Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities

Several investigators have studied the circadian rhythm of TO in rats, but very little attention has been directed toward such variations in the KH and KT activities. We studied these enzymes during a 24-hour period (see Methods) to find out if the latter two enzyme activities also vary and, if so, how such variation is related to that of the TO. Fig. VIII shows the variations in the enzyme activities for males and females. The values are expressed as per cent of the normalized activities at 9 A.M.. Tests of larger groups of animals were impractical because of timing problems. The reported results are characteristic of three different experiments with small groups however. The average 9 A.M. values are presented in Table I. For this experiment the KT assays were carried out in Im buffer with Ca^{++} present.

The diurnal variation of TO activity was characteristic of that reported previously (43, 44). No significant variations were found for the KT and KH activities for either sex.

Comparison of Tryptophan Oxygenase Activity in Normal Male and Female Rats

The effect of estrogens on TO activity is still unclear as was pointed out in the historical section. If estrogens increase this enzyme level as reported (59), a

Legend for Figure VIII

Male (240-260 g) and female (230-240 g) rats were maintained as described in Methods, with two animals of the same sex per cage. At various times during the 24-hour period two animals were sacrificed, tissue fractionation was carried out immediately, and the enzymic activities were determined. Normalized activities are expressed as a percentage of the 9 A.M. value.
o-o = male; x-x = female

Figure VIII

Diurnal Variation of Tryptophan Oxygenase, Kynurenine Aminotransferase, and Kynurenine Hydrolase

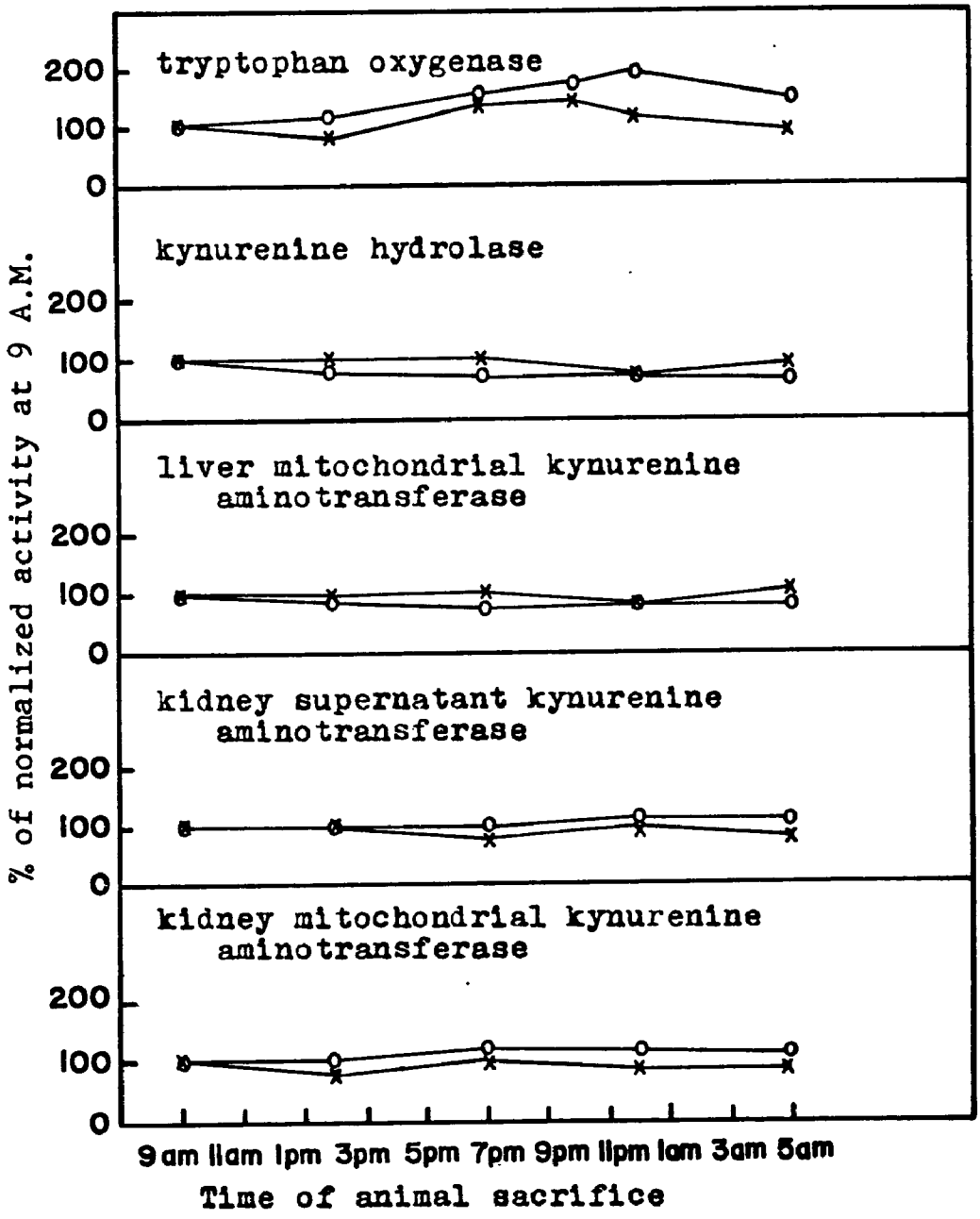


Table I

Activities of Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase at 9 A.M.

Enzyme	Male (2)[a] Specific Activity	Male (2)[a] Normalized Activity	Female (2)[a] Specific Activity	Female (2)[a] Normalized Activity
Tryptophan Oxygenase	4.4	21	6.8	25
Kynurenine Hydrolase	13.5	65	9.1	34
Kynurenine Aminotransferase				
Kidney Supernatant	34.4	29	26.4	19
Kidney Mitochondria	36.1	31	24.3	21
Liver Mitochondria	21.1	102	21.4	81

Activities are defined as described in Methods.

[a]number of animals

higher level of TO might occur in females than in males. To test this possibility a comparison of these enzyme levels in male and female rats was made. The results, reported in Table II for two weight groups, do not show a significant sex difference in the levels of TO based either on the specific activity or the normalized values.

Effect of Oral 17 β-estradiol (ES) on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

Since the most potent natural estrogen for rats is 17 β-estradiol, we tested the effect of this hormone on the TO, KH, and KT activities in male rats. The treatment and results are described in Table III. P_i buffer, pH 7.8, was used for the KH assays and the KT assays were done in P_i buffer, pH 6.4. ES treatment caused only a minor growth retardation. ES had no effect on the TO, KH, or liver mitochondrial KT activities, but caused the kidney supernatant and mitochondrial KT activities to remain at female levels.

Effects of Estradiol Benzoate (EB) Injections on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male and Female Rats

We studied the effect of subcutaneous estradiol benzoate administration on female and male rats. The KH assays were carried out in 0.05 M PP_i buffer, pH 8.0. KT assays were carried out in 0.1 M Im buffer, pH 6.5, with 10^{-3} M $CaCl_2$ present. The growth of the EB-treated

Table II

Comparison of Male and Female Tryptophan Oxygenase Activities

	Number of Animals	Body Weight	Liver Weight[a]	Specific Activity[b]	Normalized Activity[b]
Group I					
Male	6	98-144	5.6 (4.7- 6.3)	7.0 ± 0.9	31.9 ± 4.9
Female	5	79-122	5.1 (4.0- 5.8)	6.7 ± 1.6	33.2 ± 7.8
Group II					
Male	20	217-299	12.5 (10.0-15.8)	5.1 ± 0.9	24.4 ± 5.3
Female	17	217-305	9.1 (8.2-13.2)	6.0 ± 0.9	23.5 ± 3.8

Activities are defined as stated in Methods
[a]mean value with range given parentheses
[b]mean value ± standard deviation

Table III

Effects of 17 β-Estradiol on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

	Control Males (6)	ES-treated Males (5-7)	Control Females (6)
Body Weight	169 - 247	147 - 231	217 - 305
Liver Weight	9.8 (7.6-12.0)	8.8 (6.6-11.1)	11.0 (9.8-13.2)
Kidney Weight	1.8 (1.4 - 2.2)	1.5 (1.3 - 1.8)	1.8 (1.6 - 2.2)
Tryptophan Oxygenase			
Specific Activity	3.4 ± 0.7	3.2 ± 1.6	—
Normalized Activity	15.9 ± 3.3	15.3 ± 7.9	—
Kynurenine Hydrolase			
Specific Activity	6.5 ± 0.4	6.1 ± 0.6	5.5 ± 0.6
Normalized Activity	30.2 ± 2.5	28.5 ± 3.5	23.7 ± 3.2

Kynurenine Aminotransferase			
Kidney Supernatant			
Specific Activity	35.5 ± 2.0	22.5 ± 2.8[a]	23.5 ± 2.6
Normalized Activity	29.9 ± 1.4	18.3 ± 1.8[a]	16.7 ± 2.5
Kidney Mitochondrial			
Specific Activity	24.4 ± 2.7	14.6 ± 1.8[a]	14.7 ± 1.5
Normalized Activity	20.0 ± 2.5	10.8 ± 1.9[a]	10.4 ± 1.6
Liver Mitochondrial			
Specific Activity	16.8 ± 1.9	14.6 ± 0.8[b]	17.7 ± 1.9
Normalized Activity	78.7 ±10.6	68.9 ± 7.7	74.9 ± 6.6

Male rats (70 ± 5 grams) were maintained as described for normals in Methods. One-half of the group was given drinking water containing 0.5 µg ES per ml. The animals were sacrificed at various times 7 to 40 days after the treatment was begun.

The number of animals is presented in parentheses below column headings. Average organ weights are presented with the range in parentheses below. Activities are defined as stated in Methods and are presented here as mean values ± standard deviation.

[a] significantly lower than control males at $P < .001$
[b] significantly lower than control males at $P < .05$

males was only slightly retarded. The treatment and results are presented in Table IV. The TO activity of EB-treated males showed no significant difference when compared to control male activity, but the female TO activity was doubled with EB treatment. KH activity in the EB-treated males was lowered to almost that of female controls while the activity in females was unaffected by EB treatment. Kidney supernatant and mitochondrial KT activities of treated males were decreased almost to female control levels and were decreased slightly in treated female animals also. The liver mitochondrial KT activity was unaffected in either sex.

Effect of Oral L-tryptophan on Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Male Rats

Male rats were given L-tryptophan in a solid synthetic diet (see Methods). TO activity was not assayed. KH activity was assayed in P_i buffer, pH 7.8, and KT activity was assayed in P_i buffer, pH 6.4. Results appear in Table V. Oral treatment with tryptophan had no significant effect on any of the enzyme activities that were tested.

Effect of L-tryptophan Treatment by Injection on Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Male and Female Rats

These experiments were done to determine the effect of injected tryptophan on the KH and KT activities. Both males and females were tested. P_i buffer, pH 6.4, was used for the KT assays and the same buffer, pH 7.8, for

Table IV

Effect of Estradiol Benzoate on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male and Female Rats

	Control Males (6)	EB-treated Males (6)	Control Females (3)	EB-treated Females (3)
Body Weight	249 - 267	213 - 233	235 - 253	221 - 251
Liver Weight	11.6 (10.4-13.6)	9.6 (9.0-10.1)	8.9 (8.4-10.0)	9.7 (8.5-10.4)
Kidney Weight	2.2 (2.1 - 2.4)	2.1 (1.9 - 2.3)	1.9 (1.8 - 2.1)	1.8 (1.8 - 1.8)
Tryptophan Oxygenase				
Specific Activity	5.3 ± 1.4	5.7 ± 1.7	6.3 ± 0.8	11.9 ± 1.9[a]
Normalized Activity	24.1 ± 7.9	24.5 ± 3.9	23.1 ± 2.8	47.9 ± 5.8[b]
Kynurenine Hydrolase				
Specific Activity	12.1 ± 0.7	9.5 ± 0.6[c]	10.3 ± 0.9	9.6 ± 0.6
Normalized Activity	55.0 ± 4.1	41.1 ± 1.7[c]	37.7 ± 1.3	38.7 ± 0.9

Kynurenine Aminotransferase				
Kidney Supernatant				
Specific Activity	42.6 ± 3.4	31.2 + 2.4[c]	33.8 ± 0.7	30.7 ± 3.7
Normalized Activity	36.4 ± 1.8	29.8 ± 3.9[d]	26.6 ± 0.6	23.6 ± 3.9
Kidney Mitochondria				
Specific Activity	38.8 ± 1.6	20.6 ± 2.1[c]	22.5 ± 1.7	17.1 ± 1.5[e]
Normalized Activity	33.2 ± 1.2	19.6 ± 2.7[c]	17.7 ± 0.8	13.1 ± 1.5[f]
Liver Mitochondria				
Specific Activity	19.8 ± 1.4	21.3 ± 1.5	21.9 ± 1.2	22.0 ± 2.6
Normalized Activity	89.9 ± 9.8	92.5 ± 6.7	85.2 ± 9.3	88.8 ± 13.0

Male (135 ± 5 grams) and female (210 ± 5 grams) rats were maintained as described for normals in Methods. One-half of the group was injected daily with 0.1 ml of corn oil subcutaneously and the other half injected with 0.1 ml of a solution of EB in corn oil (10 μg EB daily). The animals were sacrificed at various times 16 to 30 days after treatment was begun.

The number of animals is given in parentheses below headings of the vertical columns. Average organ weights and ranges are presented. Activity values are defined as stated in Methods and presented as mean values ± standard deviation.

[a]significantly higher than control females at $P < .02$
[b]significantly higher than control females at $P < .01$
[c]significantly lower than control males at $P < .001$
[d]significantly lower than control males at $P < .01$
[e]significantly lower than control females at $P < .05$
[f]significantly lower than control females at $P < .02$

Table V

Effect of L-Tryptophan Treatment by Dietary Supplementation on Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Male Rats

	Controls (5)	Tryptophan-Fed (5)
Body Weight	214 - 338	200 - 278
Liver Weight	12.4 (10.0-14.5)	12.2 (9.3-14.3)
Kidney Weight	1.9 (1.7 - 2.2)	1.9 (1.6 - 2.2)
Kynurenine Hydrolase		
Specific Activity	6.6 ± 0.8	7.3 ± 1.2
Normalized Activity	32.2 ± 5.0	35.5 ± 2.9
Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	33.0 ± 3.3	32.7 ± 5.8
Normalized Activity	25.4 ± 3.3	25.5 ± 4.7

Kidney Mitochondria		
Specific Activity	25.3 ± 4.2	22.6 ± 3.3
Normalized Activity	19.7 ± 4.5	17.3 ± 1.5
Liver Mitochondria		
Specific Activity	16.3 ± 3.5	15.8 ± 3.3
Normalized Activity	76.8 ±13.5	77.6 ±17.1

Male rats (100 ± 5 g) were maintained on a synthetic powdered diet (see Methods) for about 5 days. After this time one-half of the group was given this diet supplemented with 10 g of L-tryptophan per kg of diet. The animals were sacrificed 1 to 37 days after treatment was begun.

The number of animals is given in parentheses below column headings. Activities are defined in Methods. Average organ weights and ranges are given.

the KH assays. The injection of L-tryptophan had no effect on any of the activities tested in either sex (Table VI).

Effect of Cortisol-21-phosphate (CP) on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

Glucocorticoids have been shown to induce TO activity. We treated male rats with a corticosteroid to further study the possibility that alterations in TO activity might result in adaptive changes in the activities of other enzymes of tryptophan metabolism. KH assays were carried out in P_i buffer, pH 7.8, and the KT assays in P_i buffer, pH 6.4. Growth was severely retarded with the 100 µg CP/ml treated rats. The expected three-fold increase in TO activity was observed with the 100 ug/ml dose of CP but not with the lower doses. There were also significant increases in the KH and liver mitochondrial KT activities at one or more of the dose levels, but at none of the doses tested did the kidney KT activities change significantly (Tables VII-IX).

Effect of Oral Pyridoxine HCl on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Female Rats

As mentioned in the historical section, pyridoxine deficiency has profound effects on tryptophan catabolism. The percent saturation of KH and KT with PLP declines during such deficiencies and the apparent levels of apoenzyme

Table VI

Effect of L-Tryptophan Treatment by Injection on Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Male and Female Rats

	Control Males (3-6)	Try-Inj. Males (3-7)	Control Females (6)	Try-Inj. Females (3-6)
Body Weight	223 - 285	152 - 246	194 - 210	198 - 210
Liver Weight	12.2 (10.1-13.6)	10.1 (6.3-12.2)	8.6 (7.9 - 9.4)	8.7 (8.5 - 9.0)
Kidney Weight	2.1 (1.8 - 2.4)	1.8 (1.3 - 2.1)	1.5 (1.4 - 1.6)	1.6 (1.5 - 1.7)
Kynurenine Hydrolase				
Specific Activity	6.2 ± 1.0	5.1 ± 0.7	5.7 ± 0.6	6.0 ± 0.5
Normalized Activity	30.0 ± 3.2	24.7 ± 6.1	24.4 ± 3.0	26.1 ± 1.0
Kynurenine Aminotransferase				
Kidney Supernatant				
Specific Activity	38.9 ± 4.5	42.6 ± 3.5	29.1 ± 4.4	31.1 ± 1.5
Normalized Activity	31.6 ± 3.6	34.8 ± 4.2	21.5 ± 3.7	24.2 ± 2.2

Kidney Mitochondria				
Specific Activity	25.1 ± 3.6	24.4 ± 2.9	18.5 ± 0.9	19.3 ± 3.7
Normalized Activity	20.4 ± 2.5	20.1 ± 3.0	13.7 ± 1.4	15.0 ± 1.2
Liver Mitochondria				
Specific Activity	14.2 ± 3.8	12.9 ± 3.3	18.6 ± 2.3	19.0 ± 0.9
Normalized Activity	67.9 ±18.2	61.0 ±15.6	78.9 ± 8.7	82.2 ± 3.8

Male and female rats (100 ± 5 g) were maintained as described for normals in Methods. One-half of each group was injected intraperitoneally daily with 25 mg of L-tryptophan per 100 grams body weight in mls of water solution. Controls received the same volume of distilled water. The animals were killed at various times 7 to 30 days after treatment was begun.

Table VII

Effect of Cortisol-21-Phosphate (100µg/ml) on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

	Controls (5-6)	CP-treated (5-6)
Body Weight	225 - 305	174 - 206
Liver Weight	11.8 (10.7-13.5)	8.8 (7.3-10.4)
Kidney Weight	2.0 (1.7 - 2.1)	1.6 (1.4 - 1.8)
Tryptophan Oxygenase		
Specific Activity	6.1 ± 0.9	21.6 ± 3.9[a]
Normalized Activity	27.6 ± 3.3	99.6 ±17.5[a]
Kynurenine Hydrolase		
Specific Activity	6.5 ± 1.2	9.0 ± 1.1[b]
Normalized Activity	29.7 ± 4.6	41.7 ± 4.0[c]

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	38.4 ± 2.0	37.0 ± 2.3
Normalized Activity	28.7 ± 2.4	31.6 ± 2.7
Kidney Mitochondria		
Specific Activity	34.0 ± 4.6	28.1 ± 3.0[d]
Normalized Activity	25.8 ± 2.6	24.0 ± 3.2
Liver Mitochondria		
Specific Activity	18.0 ± 2.2	24.1 ± 2.1[c]
Normalized Activity	81.5 ±10.6	114.6 ± 2.6[a]

Male rats (150-170 g) were maintained as described for normals (see Methods). Experimental animals were given drinking water containing 100 μg of CP/ml. The animals were sacrificed at various times 5 to 26 days after treatment was begun.
All quantities are defined as in previous tables.

[a] significantly elevated above controls at $P < .001$
[b] significantly elevated above controls at $P < .02$
[c] significantly elevated above controls at $P < .01$
[d] significantly lower than controls at $P < .02$

Table VIII

Effect of Cortisol-21-Phosphate (50µg/ml) on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

	Controls (4)	CP-treated (4)
Body Weight	228 - 330	203 - 289
Liver Weight	12.1 (10.6-13.9)	10.5 (9.0-11.6)
Kidney Weight	2.1 (1.8 - 2.3)	1.9 (1.6 - 2.3)
Tryptophan Oxygenase		
Specific Activity	4.0 ± 1.2	6.2 ± 2.6
Normalized Activity	17.5 ± 4.7	26.9 ±16.9
Kynurenine Hydrolase		
Specific Activity	6.8 ± 0.4	9.3 ± 0.6[a]
Normalized Activity	30.1 ± 1.2	40.0 ± 5.5[b]

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	39.9 ± 2.8	38.2 ± 3.6
Normalized Activity	30.5 ± 2.4	30.0 ± 3.4
Kidney Mitochondria		
Specific Activity	24.1 ± 2.4	24.4 ± 2.8
Normalized Activity	18.5 ± 2.2	19.2 ± 2.3
Liver Mitochondria		
Specific Activity	16.5 ± 0.5	20.6 ± 3.2
Normalized Activity	73.5 ± 3.2	88.0 ±14.8

Male rats (150-170 g) were maintained as described for normals (see Methods). Experimental animals were given drinking water containing 50 μg CP/ml. The animals were sacrificed at various times 5 to 26 days after treatment was begun. All quantities are defined as in previous tables.

[a]significantly elevated above controls at $P < .001$
[b]significantly elevated above controls at $P < .05$

Table IX

Effect of Cortisol-21-Phosphate (10µg/ml) on Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Male Rats

	Controls (13-14)	CP-treated (13)
Body Weight	198 - 337	231 - 323
Liver Weight	13.0 (8.7-16.6)	12.0 (10.9-13.6)
Kidney Weight	2.2 (1.6 - 2.7)	2.1 (1.8 - 2.6)
Tryptophan Oxygenase		
Specific Activity	5.4 ± 1.2	6.3 ± 0.9
Normalized Activity	25.2 ± 6.4	27.4 ± 6.7
Kynurenine Hydrolase		
Specific Activity	7.0 ± 0.6	8.7 ± 1.1[a]
Normalized Activity	32.4 ± 2.7	37.6 ± 3.8[a]

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	35.9 ± 2.2	37.0 ± 3.3
Normalized Activity	28.4 ± 2.3	28.6 ± 2.4
Kidney Mitochondria		
Specific Activity	25.2 ± 4.6	25.0 ± 3.3
Normalized Activity	19.7 ± 3.4	19.4 ± 2.9
Liver Mitochondria		
Specific Activity	18.8 ± 3.8	20.9 ± 4.5
Normalized Activity	86.8 ±17.6	90.4 ±18.6

Male rats (150-170 g) were maintained as described for normals (see Methods). Experimental animals were given drinking water containing 10 μg CP/ml. The animals were sacrificed at various times 5 to 26 days after treatment was begun.

All quantities are defined as in previous Tables.

[a]significantly higher than control values at $P < .001$

also decline. The purpose of the present experiment was to determine whether prolonged supplementation with excessive amounts (1 mg per day intraperitoneally) of pyridoxine might alter the percent saturation and apoenzyme levels of KT, KH, or TO. The KT assays of pyridoxine-treated and control rats were done in Im buffer, pH 6.5, containing Ca^{++}. The KT assays were done in PP_i buffer, pH 8.0. No significant differences were found in the activity levels or percent saturation values of any of the three enzymes in pyridoxine-injected female rats as compared with the water-injected controls (Table X).

Comparison of Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Castrated and Sham-operated Male Rats

Little is known about the effects of androgens on tryptophan metabolism. It is possible that some of the observed estrogen effects may be mediated by antagonism of androgen production or function. In the present study, male rats were castrated or sham-operated at about 95 gms body weight. After about 6 weeks the TO, KH, and KT activities of the two groups were compared. The KT assays were done in Im buffer, pH 6.5, containing 1 mmolar $CaCl_2$ and the KH assays were done in PP_i buffer, pH 8.0. Castration caused a significant decrease in KH and kidney mitochondrial KT activities. The TO, kidney supernatant KT, and liver mitochondrial KT activities were not significantly altered (Table XI).

Table X

Effect of Pyridoxine Administration on the Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Female Rats

	Controls (5)	B_6-treated (7)
Body Weight	146 - 167	146 - 193
Liver Weight	7.5 (6.9 - 8.3)	8.4 (7.1-10.3)
Kidney Weight	1.4 (1.3 - 1.6)	1.6 (1.4 - 1.8)
Tryptophan Oxygenase		
Specific Activity	6.5 ± 0.9	6.3 ± 0.6
Normalized Activity	31.1 ± 5.8	31.3 ± 4.2
Kynurenine Hydrolase		
Specific Activity	8.5 ± 0.5	8.4 ± 1.0
Normalized Activity	40.6 ± 3.0	41.6 ± 3.7
% Saturation	62.1 ± 2.3	63.5 ± 3.0

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	27.6 ± 1.7	28.1 ± 6.6
Normalized Activity	24.8 ± 1.4	25.9 ± 4.7
% Saturation	59.5 ± 2.8	64.7 ±12.6
Kidney Mitochondria		
Specific Activity	19.7 ± 1.7	20.1 ± 3.5
Normalized Activity	17.7 ± 1.2	18.6 ± 2.6
% Saturation	69.2 ± 4.7	70.1 ± 5.2
Liver Mitochondria		
Specific Activity	17.8 ± 1.6	17.2 ± 1.3
Normalized Activity	85.2 ± 8.9	85.9 ±10.0
% Saturation	76.6 ± 2.7	80.1 ± 3.6

Female rats (90-100g) were maintained as described for normal animals. Control animals were injected intraperitoneally with 0.5 ml distilled water at 4 P.M.-5 P.M. daily. The other animals were injected with 0.5 ml of various pyridoxine solutions resulting in doses of 1 to 3 mg of pyridoxine per rat. The animals were sacrificed 9 to 16 days after treatment was begun. % saturation is defined as the activity obtained in the absence of added PLP divided by the activity obtained in the presence of added PLP multiplied by 100.

Table XI

Comparison of Tryptophan Oxygenase, Kynurenine Hydrolase, and Kynurenine Aminotransferase Activities in Castrated and Sham-operated Male Rats

	Sham-Operated (4)	Castrated (4)
Body Weight	335 - 369	330 - 346
Liver Weight	15.1 (13.4-17.2)	13.6 (12.7-14.4)
Kidney Weight	2.9 (2.8 - 3.0)	2.6 (2.4 - 2.8)
Tryptophan Oxygenase		
Specific Activity	7.8 ± 1.2	6.4 ± 1.2
Normalized Activity	32.8 ± 2.8	25.7 ± 4.2
Kynurenine Hydrolase		
Specific Activity	13.2 ± 0.4	11.4 ± 1.1[a]
Normalized Activity	56.1 ± 2.2	46.1 ± 4.6[b]

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	39.2 ± 1.9	39.3 ± 6.4
Normalized Activity	32.2 ± 2.3	33.3 ± 9.1
Kidney Mitochondria		
Specific Activity	43.1 ± 4.8	31.2 ± 5.2[a]
Normalized Activity	35.5 ± 4.9	24.3 ± 5.8[a]
Liver Mitochondria		
Specific Activity	22.3 ± 2.3	21.4 ± 2.6
Normalized Activity	94.5 ± 4.8	87.5 ± 6.4

Male rats (95 ± 5 g) were anesthetized with ether. The testes were removed in the experimental animals. Incisions were made in a similar manner in the sham-operated animals, but the testes were not removed. Animals were sacrificed at various times 43 to 48 days after the operations.

[a]significantly lower than sham-operated at $P < .05$
[b]significantly lower than sham-operated at $P < .01$

Effect of Testosterone Propionate (TP) on the Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Female Rats

To further explore the role of androgens in tryptophan metabolism, we injected testosterone propionate (1 mg in 0.1 ml corn oil intramuscularly per day) into female rats (initially 90 to 100 gms body weight). After 5 to 18 days the androgen-treated females and corn oil-injected controls were sacrificed and the KH and KT activities were determined. The KT assays were carried out in Im buffer, pH 6.5, containing $CaCl_2$. KH assays were done in PP_i buffer, pH 8.0. The kidney KT activities were increased substantially by TP-treatment whereas the liver KT and KH activities were not significantly altered (Table XII). In addition, the percent saturation values of the TP-treated females were significantly decreased for the kidney supernatant KT. Per cent saturation is defined as the activity obtained in absence of added PLP divided by the activity obtained in the presence of added PLP and multiplied by 100.

Table XII

Effect of Testosterone Propionate on Kynurenine Hydrolase and Kynurenine Aminotransferase Activities in Female Rats

	Controls (6)	TP-treated (6)
Body Weight	138 - 180	146 - 206
Liver Weight	8.2 (6.2 - 9.4)	8.2 (6.4 - 8.9)
Kidney Weight	1.5 (1.2 - 1.7)	1.7 (1.2 - 2.0)
Kynurenine Hydrolase		
Specific Activity	8.3 ± 0.5	9.3 ± 1.5
Normalized Activity	40.1 ± 5.1	41.7 ± 6.4
% Saturation	60.0 ± 2.0	59.0 ± 3.0

Kynurenine Aminotransferase		
Kidney Supernatant		
Specific Activity	27.6 ± 1.9	35.0 ± 3.7[a]
Normalized Activity	25.3 ± 3.0	33.4 ± 5.5[b]
% Saturation	60.1 ± 4.5	52.5 ± 1.2[a]
Kidney Mitochondria		
Specific Activity	16.3 ± 1.7	24.4 ± 3.1[c]
Normalized Activity	14.9 ± 1.7	23.3 ± 4.3[a]
% Saturation	73.3 ± 5.0	70.4 ± 2.4
Liver Mitochondria		
Specific Activity	16.7 ± 2.4	18.7 ± 2.1
Normalized Activity	81.1 ±14.3	84.1 ± 8.5
% Saturation	79.7 ± 3.6	82.7 ± 5.9

Female rats (90-100 g) were maintained as described for normals (see Methods). Control animals were injected daily intramuscularly with 0.1 ml of corn oil, and the treated animals with 0.1 ml of corn oil containing 1 mg of testosterone propionate. The animals were sacrificed 5 to 18 days after treatment was begun.

[a]significantly different from control values at $P < .01$
[b]significantly higher than control values at $P < .02$
[c]significantly higher than control values at $P < .001$

DISCUSSION

As stated in the introduction a major focus of this thesis was to study the effects of estrogens on tryptophan metabolism. We have studied the effects of estrogens on the TO, KH, and KT activities to find out if variations in the activity levels of these enzymes might explain the abnormally high excretion rates of certain tryptophan metabolites that result from estrogen treatment. We have also run several experiments to evaluate the validity of some hypothesized mechanisms by which estrogens affect tryptophan metabolism.

Results from the diurnal variation studies showed a two-fold diurnal increase in the TO activity of male rats with maximal activity near midnight (Fig. VIII). This pattern is in accordance with previous reports (43, 44). There had been no previous reports of such a study with female rats. This study was designed to test whether there was a sex difference in the diurnal rhythm of TO activity that might be related to the estrogen effects on tryptophan metabolism. We found that the diurnal rhythm of TO in females was similar to that in males although there were indications that the magnitude and time of maximal activity may not be identical in the two sexes (Fig. VIII).

We found that neither KH nor KT activities showed any significant changes for either sex over the 24 hour period. In contrast, Hardeland (69) found marked changes in both the KT and KH activities. All of his specific activities

were lower than those reported here. He used DL-kyn rather than the L-isomer, and apparently at concentrations 1/15 of that of the L-form used in our assays. His system probably did not saturate the enzyme with L-kyn thus making interpretation of his results difficult.

The diurnal studies also allow one to make comparisons of the relative maximal rates of synthesis and degradation of kyn at various times of the day (Fig. IX), using the assumption that the activities at pH 7.4 represent approximations of the maximal rates of formation and degradation of kyn *in vivo*. Needless to say, such comparisons serve only roughly to outline the dimensions of the regulation problem and do not provide evidence that such rates do occur *in vivo*.

Fig. IX compares the variation of the liver enzymes diurnally. The activities have all been adjusted to the *in vitro* activities at pH 7.4 by multiplying the known ratios of the activities at pH 7.4 to those measured at the optimum pH values. If one assumes that these rates represent maximal values for *in vivo* conditions, it can be surmised that the potential rate of kyn or 3OHkyn degradation by the two alternate paths would normally exceed the potential rate of kyn formation from tryptophan throughout the day in both males and females. Since kyn does not accumulate to a large degree normally in urine (70), this inference is consistent with *in vivo* observations. It should be noted, however, that in males the potential rate

Legend for Figure IX

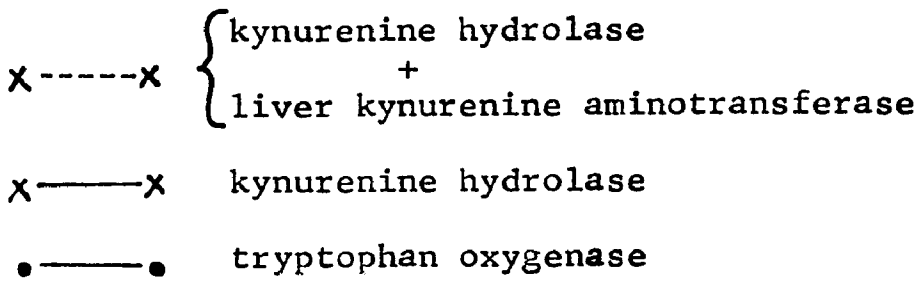
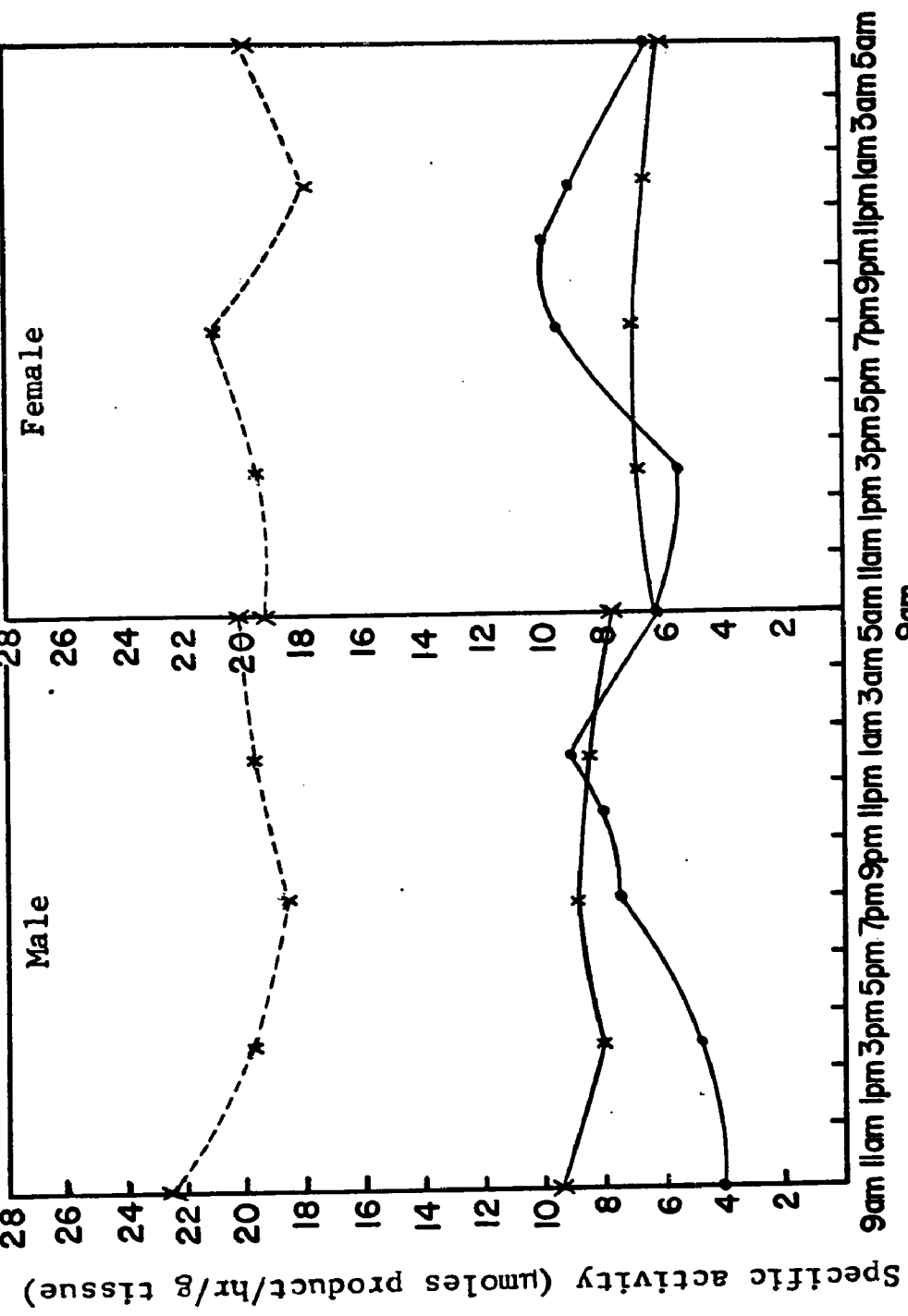


Figure IX

Specific Activity of Tryptophan Oxygenase, Kynurenine Hydrolase, and Liver Mitochondrial Kynurenine Aminotransferase Adjusted to pH 7.4



of degradation of kyn or 3OHkyn by KH alone would normally exceed or equal the potential rate of formation at all times. In females, the lower potential KH activity relative to the TO activities would provide less reserve for handling maximal kyn production especially at night. One might, therefore, conclude that the liver KT of females would more likely be brought into play to handle the excess kyn produced under conditions causing maximal TO activity. Whether these potentialities are actually expressed *in vivo* is not known. It has been reported in human subjects that the accumulation of metabolites is much greater if tryptophan loads are given when the TO level is high (43). Michael *et al.* (71) have also reported that females given L-tryptophan excreted a substantially greater portion of the dose (14.8%) in the urine as metabolites than males (6.5%) given the same dose per kg body weight. No such differences were seen between male and female children or between adult males and children. These results may be related to the development of human sex differences in the liver KH levels similar to those seen in rats. However, such *in vitro* data are not available for human subjects.

When kyn formation is accelerated 5 to 10-fold by the induction of TO following tryptophan administration, it is obvious that the alternate pathway via KT must metabolize the excess kyn if accumulation of kyn is to be avoided. The accumulation of the KT products, KA and XA, is in fact characteristic of the excretion patterns of tryptophan-

loaded rats and human subjects (72).

Our results (Fig. IX) indicate that the potential liver KT activity exceeds the potential KH activity as measured at pH 7.4 or lower pH values. It would appear, therefore, that the potential KT activity is normally not as fully expressed as the KH activity, since the major pathway is via KH. The mitochondrial membrane may act as a barrier to substrate penetration *in vivo* in a fashion similar to that observed by Mason *in vitro* (see Methods). Another possibility is that the K_m for kyn may be greater for KT than for KH *in vivo*. Data pertinent to this possibility will be presented in Part II.

The kidney KTs may normally have only minimal activity *in vivo* because the amount of kyn presented to the kidney depends in part on the amount escaping catabolism in the liver. A mean serum concentration of approximately 3 µg kyn per ml has been reported for normal human subjects (73). Serum levels for rats have not been reported. The kidney KTs may be more important, however, when the liver KH and KT cannot metabolize all of the kyn produced under such conditions as B_6-deficiency, estrogen treatment, or tryptophan administration.

Up until the present time there had been two reports concerning a sex difference in the TO activity levels of normal male and female rats and these reports contradicted each other. One group (74) reported that adult female rats have a higher TO activity than adult males but the other

group (75) reported no significant difference. Our results (Table II) confirm the latter report. The specific activity levels of the adult females tended to be slightly higher than those of the adult males but total and normalized activities showed no such differences. The livers of adult female rats tended to be smaller than the livers of adult males of the same body weight (Table II, Fig. VII). This cancelled out the effect of the higher specific activity of the females when total or normalized activity was calculated (see Methods). It was pointed out by Rose (59) that the earlier study which found a sex difference in TO activity levels did not use added heme cofactor in the assays and therefore did not assay a fully activated system. Thus it would appear that normal male and female rats do not exhibit a sex difference in TO activity levels such as that observed for KH and kidney KT levels.

After finding that normal male and female rats show no sex difference in TO activity levels the next question to be answered was whether exogenous estrogens might affect TO activity levels. The various estrogen treatment experiments enabled us to answer this question. In addition these experiments allowed us to look at the TO, KH, and KT activities simultaneously during treatment and to determine the effect of estrogen on each enzyme relative to the others. Neither the oral (Table III) nor the injected (Table IV) estrogen had any significant effect on TO activity levels in male rats. In contrast, the injection

of estradiol benzoate resulted in a two-fold increase in TO activity of adult female rats. This latter finding is in agreement with a preliminary report by Rose (56).

KH activity levels of EB-injected male rats remained at normal female levels (Table IV) but oral estradiol administration (Table III) did not affect KH levels. One explanation for the different responses to oral and injected estrogens may lie in the probability that oral administration presented a lower dose of hormone to the organs than the injection method. The rats drank 30 to 40 mls of water daily and, therefore, received approximately 15 to 20 µg of estrogen daily (see legend for Table III). Absorption of estradiol administered orally is known to be poor, so those animals were probably receiving less estrogen than the injected animals, which were receiving 10 µg daily of estradiol benzoate.

We mentioned in the introduction that very little is known about the effect of tryptophan on tryptophan metabolism even though tryptophan-load tests were widely used for diagnostic purposes. The abnormally high excretion rates of pregnant or estrogen-treated women and animals are also determined after a tryptophan-load has been administered. Therefore, it was important to know if tryptophan itself had any effect on the enzymes that are affected by estrogens. It has been well established that tryptophan increases TO activity levels in rats. L-tryptophan had no effect on the KH or KT activities at the

levels tested (Tables V and VI).

The tryptophan, cortisol, and B_6 treatment experiments were devised to help us evaluate some of the proposed mechanisms by which estrogens might affect tryptophan metabolism. It has been suggested by Rose (59) that the abnormally high excretion rates of certain tryptophan metabolites associated with pregnancy or estrogen treatment may be the direct result of elevated TO activity. He proposed that the ability of estrogens to cause an increase in circulating glucocorticoid levels (63) could result in increased TO activity levels without compensating changes in KT and KH activities, thus causing kyn accumulation. Although our results (Table IV) are consistent with this suggestion, they also indicate that the response of TO to estrogens may be limited to non-physiological doses of estrogens. There was no evidence of a sex difference in TO levels (Table II) although elevated corticosteroid levels have also been observed in females (64).

Since estrogen levels do influence the circulating corticosteroid levels, it was important to test whether the observed effects of estrogens on KH and KT activities and estrogen-dependent sex differences might be mediated by corticosteroids. Cortisol-21-phosphate treatment at 100 µg per day resulted in a four-fold elevation in TO activity but did not change kidney KT activities. Lower doses (50 µg, 10 µg) also did not affect kidney KT activities. TO induction is presumably accompanied by increased rates

of tryptophan catabolism. A more direct way of increasing tryptophan catabolism is to administer supplemental tryptophan, which induces TO as well as providing additional substrate. Long-term supplementation of either male or female rats with tryptophan did not significantly alter kidney KT activities (Tables V and VI).

Thus, treatments that increased TO activity and tryptophan catabolism were not accompanied by decreased KT activities and treatments or conditions that decreased KT activities were not accompanied by altered TO activities. It appears safe to conclude, not withstanding the interactions of estrogens and corticosteroid metabolism, that corticosteroids and estrogens can operate independently in controlling the activities of TO, KH, and KT.

As mentioned in the Introduction, pyridoxine deficiency caused elevated rates of excretion of tryptophan metabolites similar to those caused by estrogen administration. In both cases pyridoxine administration corrected the abnormality. The B_6-deficiency is generally assumed to selectively deplete the KH pathway by depriving KH of PLP, thus diverting catabolism via the KT pathway which is less affected by PLP depletion. Two hypotheses have been presented to account for the ability of estrogen treatment to cause effects that mimic those of B_6-deficiency. According to one (49, 59), estrogens act in their conjugated form to interfere with PLP binding *in vivo* as they were reported to do *in vitro* (57, 58). Pyridoxine administration would

correct the resulting relative deficiency of PLP by providing precursor for additional PLP synthesis. According to the other hypothesis, estrogens cause increased corticosteroid levels which induce TO. Increased TO activity results in an increased rate of formation of kyn which accumulates, saturates the KH pathway, and is partially diverted to the KT pathway. Pyridoxine administration, according to this view, would accelerate the KH pathway and prevent metabolic accumulation by providing additional PLP to fully saturate the KH.

Both of the above hypotheses assume incomplete saturation of KH with PLP as an explanation of the corrective effect of administered pyridoxine. Our experiments have consistently shown percent saturation values for KH of approximately 60% which indicates some potential for activation by additional PLP. However, the administration of a substantial excess of pyridoxine (Table X) did not significantly alter this value. Additional experiments with varied pyridoxine dosages at various times preceeding sacrifice will be required to test the ability of supplemental pyridoxine to increase the saturation of KH with PLP.

A role of PLP levels in controlling the kidney KT apoenzyme levels has been indicated by the decline in activity during B_6-deficiency (60) and by the observation that PLP stabilized the enzyme <u>in vitro</u> (58). The daily administration of pyridoxine did not elevate the KT activities (Table X) of female rats, so it appears unlikely

that the lower KT levels in females are due to a relative deficiency in PLP. Accurate measurements of PLP and pyridoxamine phosphate levels of rat kidneys will be required to further assess the potential role of this coenzyme in controlling KT levels.

Our experiments (Table III) show for the first time that treatment of male rats with a natural estrogen, 17 β-estradiol, causes them to develop kidney KT activities similar to those of normal females. They also show (Table XI) that castration of male rats also causes depressed enzyme levels, whereas injection of testosterone propionate causes the female KT levels to increase to male levels. These findings raise the question of which hormone, estrogen or androgen, is most directly responsible for the observed effects on KT activities. It is possible that estrogen treatment acted by inhibiting the production or function of testosterone. This potential was obvious in the experiment in which estradiol benzoate was injected; the average testicular weight of EB-treated males was less than one-half that of the controls.

Although it is not clear which gonadal steroid acts most directly, there are reasons to suspect that it might be testosterone. Testosterone, but not estradiol, is known to be a trophic agent for kidney (76). Acceleration of enzyme production as indicated for the testosterone effect is characteristic of steroid hormone action whereas decreased enzyme production as indicated for the estrogen

effect is atypical.

Some interaction of testosterone and PLP in controlling KT activity is indicated by the lower percent saturation of the kidney supernatant KT with PLP in the TP-treated females. Normal male rats also had a lower value than normal females (60), so the effect is probably physiologically significant. While this effect suggests that testosterone-treated females and normal males may have a lower kidney cytosol PLP concentration than normal females, a very recent report (77) claims that normal male rats actually have a higher kidney PLP concentration than castrated males. Pyridoxine administration did not increase the PLP levels of the castrate kidneys unless testosterone was also provided. The authors also observed a decrease in the pyridoxine phosphate oxidase activity level in castrates which was prevented by testosterone administration. They assumed that the low oxidase activity limited PLP production in the kidneys of the pyridoxine-treated castrates. Further study of pyridoxine metabolism in kidney may delineate the observed interactions of testosterone and PLP.

Part II

IN VITRO STUDIES

INTRODUCTION

In Part I we have described sex differences and hormonally-induced differences in KT, KH, and TO activities that are most likely reflections of differences in enzyme concentrations. Part II describes studies that were initiated prior to the whole-animal studies to determine those characteristics of the measured enzymes that must be known in order to make reliable activity measurements. These studies were extended in an attempt to discover enzyme characteristics that might have regulatory significance. These latter studies have been restricted largely to KH and KT because the properties of TO have already been extensively investigated.

RESULTS

Validity of the Assay Systems

In measurements of KT activity vs time, using conditions described in Methods, significant departure from linearity was detected at KA concentrations exceeding 0.41 μmoles per incubation mixture (0.6 ml). Using enzyme concentrations that gave KA concentrations below this value, a linear relationship of KA concentration to volume of each enzyme fraction was obtained. Similar studies of KH demonstrated a linear relationship of fraction volume to enzyme activity when AA concentrations did not exceed 0.41 μmoles per incubation mixture (1.2 ml).

The linear responses described above provide some assurance that the assay systems will measure enzyme activities accurately. In addition, it should be recognized that the fractionation procedure and assay conditions effectively eliminate competition between the KH and KT reactions. KH activity resides exclusively in the supernatant fraction of liver and KT in the mitochondria. At their respective optimal pH's, 6.4 for KT and 8.0 for KH, the competing enzyme is inactive. The KT assay system measures both kyn loss and KA formation, so significant loss of substrate via competing reactions would be detected.

The potential loss of kyn via hydroxylation to 3OHkyn by the hydroxylase of the outer mitochondrial membrane was considered. This reaction of intact mitochondria requires NADPH as a cofactor and cannot utilize that produced in the

mitochondria (78). As an additional test of the validity of the assay methods, the incubation mixtures have been chromatographed directly on microcrystalline cellulose thin-layer plates using a methanol:butanol:benzene:water solvent in a volume ratio of 2:1:1:1. Since all of the immediate products of kyn catabolism fluoresce intensely under U.V. light (79), any significant interference by competing reactions would have been detected. In such chromatographs of the products of the TO, KH, and KT assay systems, the only fluorescing compounds detected were the appropriate products, i.e. kyn, AA, and KA respectively.

Properties of Kynurenine Aminotransferases

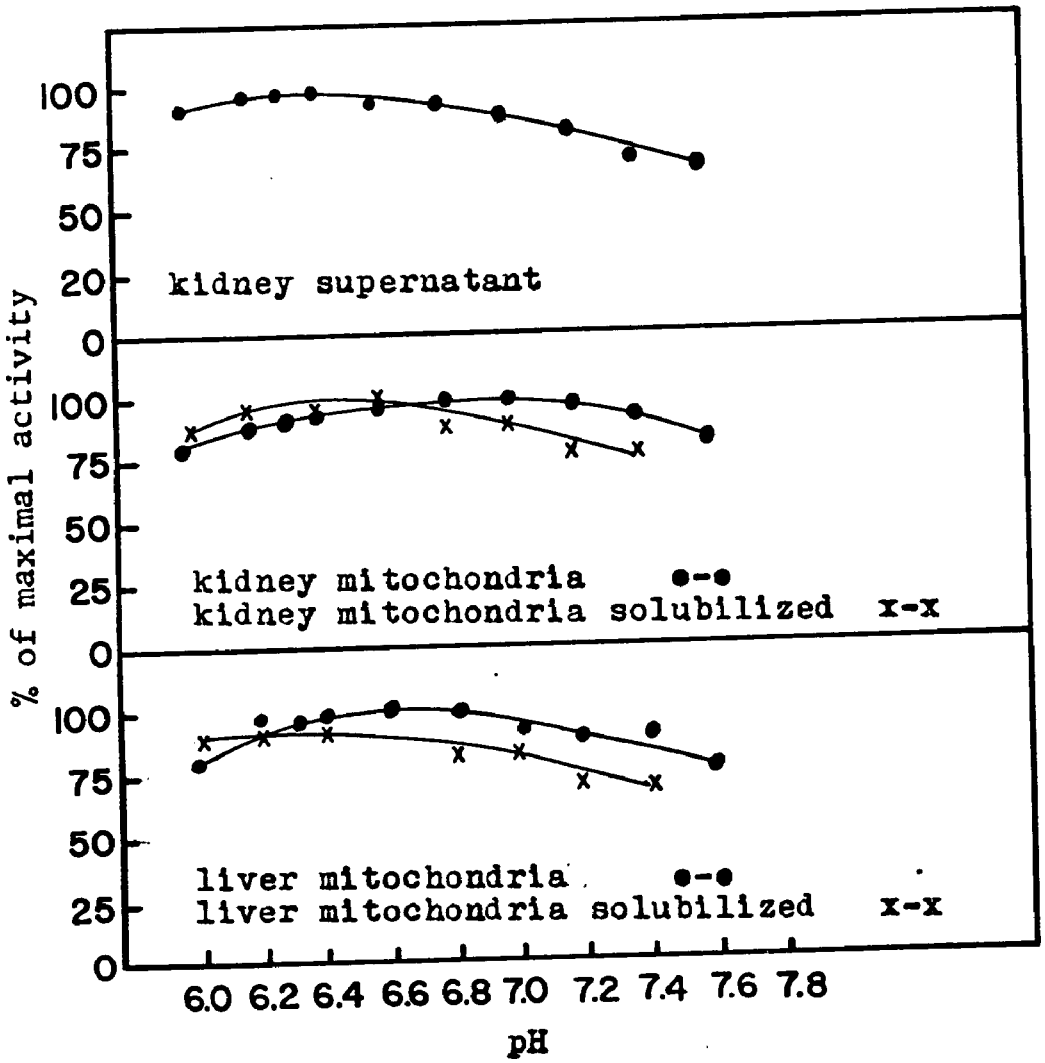
Determination of pH optima of the supernatant and mitochondrial fractions Previous investigators have reported pH optima of KT in whole homogenate systems. It was necessary to determine the optima of the supernatant and mitochondrial KT's. This study also allowed us to look for differences in the KT enzymes that might account for differences in their response to dietary and hormonal treatments. The incubations were carried out in 0.05 M P_i buffer of varying pH values as described in the methods section for the KT assays. The data show pH optima of 6.4-6.6 for the supernatant and solubilized mitochondrial kidney KT and for both the intact mitochondrial and solubilized mitochondrial liver KT. The pH optimum of the intact kidney mitochondrial KT was near 7.0 (Fig. X).

Legend for Figure X

The incubation mixture (0.6 ml) contained 0.05 M P_i buffer of various pHs, 0.1 mM PLP, 5 mM αkg, 3 mM kynSO_4, and 0.1 ml of enzyme fraction (equivalent to 20 mg wet weight of tissue). The mixture was incubated at 37° for 30 minutes.

Figure X

pH Optima of the Supernatant and Mitochondrial Kynurenine Aminotransferases



Data collected later by Mason (unpublished experiments) for Im buffer containing Ca^{++} showed a pH optimum of 6.4-6.6 for all the KT's including the intact kidney mitochondrial KT. The higher pH optimum found for that fraction with P_i buffer appeared to be a result of restricted substrate penetration at the lower pH values.

Substrate and coenzyme saturation kinetics of crude preparations The determination of appropriate assay conditions requires some knowledge of the concentrations of substrate and coenzyme necessary for saturation of the enzyme. The studies reported here were done to supply this information for the KT fractions. The differences in responses of the KT fractions to estrogen treatment and B_6-deficiency could be due to differences in enzyme properties. Therefore, we compared the saturation kinetics of crude enzyme preparations in an attempt to discover any characteristics that were strikingly different. The fractions were prepared as described in the methods section. Endogenous PLP was apparently removed from the mitochondrial KT by treating the mitochondrial fractions with 0.075 M P_i at 37° for 15 minutes. After centrifuging and resuspending the mitochondria in the original volume of sucrose, KT activity was measured. Activity was found only in the presence of added PLP. The kidney supernatant fraction was frozen and thawed several times to remove endogenous PLP from the enzyme. Both P_i treatment and freezing and thawing had previously

been found to cause such loss of activity in homogenates (80). The loss was assumed to be due to dissociation of the PLP from the enzyme because activity was recovered when PLP was added. Assays were carried out in 0.1 M Im buffer, pH 6.5, with Ca^{++} present. Characteristic reciprocal plots for the enzyme fractions are presented for kyn sulfate, αkg, αka, and PLP in the Appendix (Fig. XIII-XVI respectively). The apparent K_m values for the enzyme fractions are given in Table XIII. The K_m values were calculated from the intercepts of the reciprocal plots on the 1/substrate axis. The intercept is eqivalent to $-1/K_m$ according to Michaelis-Menten kinetics.

Table XIII

Apparent K_m Values of Crude Kynurenine Aminotransferase Fractions for Substrates and Coenzyme

Substrate or Coenzyme	Kidney Supernatant	Kidney Mitochondria	Liver Mitochondria
$KynSO_4$(mM)	3.12	3.45	3.20
αkg(mM)	0.77	1.89	0.91
αka(mM)	1.00	1.45	2.00
PLP(μM)	0.68	1.84	0.77

Substrate and coenzyme saturation kinetics of partially-purified fractions A more direct comparison of the properties of the three KT enzymes required the solubilization of the

mitochondrial enzymes of liver and kidney. For these experiments frozen fractions were used. The thawed mitochondrial fractions were centrifuged at 30,000 g for 20 minutes and the supernatants, containing the solubilized enzyme, were used. The fractions were partially purified by a heat treatment at 55^{o} for 5 minutes in the presence of 10^{-3} M PLP followed by precipitation of the resulting supernate fraction at 40% to 70% saturation with ammonium sulfate. These treatments resulted in a 4 to 10-fold purification of the enzyme fractions. These fractions were used for the saturation studies. The conditions were the same as those used for the crude enzymes.

Fractions used for the PLP saturation studies were prepared without the heat step because it was very difficult to remove PLP from the KT once it had been introduced at the 10^{-3} M concentration. The assay conditions were the same as those previously used in the PLP studies of the crude fractions. The apparent K_m values for the partially purified enzymes are presented in Table XIV (Fig. XVII-XX) in the Appendix.

Table XIV

Apparent K_m Values of Substrates and Coenzyme for Partially-purified Kynurenine Aminotransferase Fractions

Substrate or Coenzyme	Kidney Supernatant	Kidney Mitochondria	Liver Mitochondria
kynSO_4(mM)	3.12	3.12	3.12
αkg(mM)	0.63	0.91	0.71
αka(mM)	2.00	1.25	1.43
PLP(μM)	0.59	1.20	1.20

Phosphate inhibition As mentioned above, the KT activity of kidney homogenates is lost during incubation with P_i buffer and can be recovered by adding PLP. We tested whether P_i inhibited each of the three KT enzyme fractions and compared the rates of inactivation of each in P_i buffer (Fig. XI). The fractions, prepared in the usual manner, were incubated at 37° in 0.1 M P_i buffer, pH 6.4, for various time periods. They were then assayed in the presence and absence of added PLP. Data for intact mitochondrial fractions are presented. No significant differences were noted. All fractions showed a rapid and complete decay of activity that could be fully recovered by adding PLP (10^{-4} M).

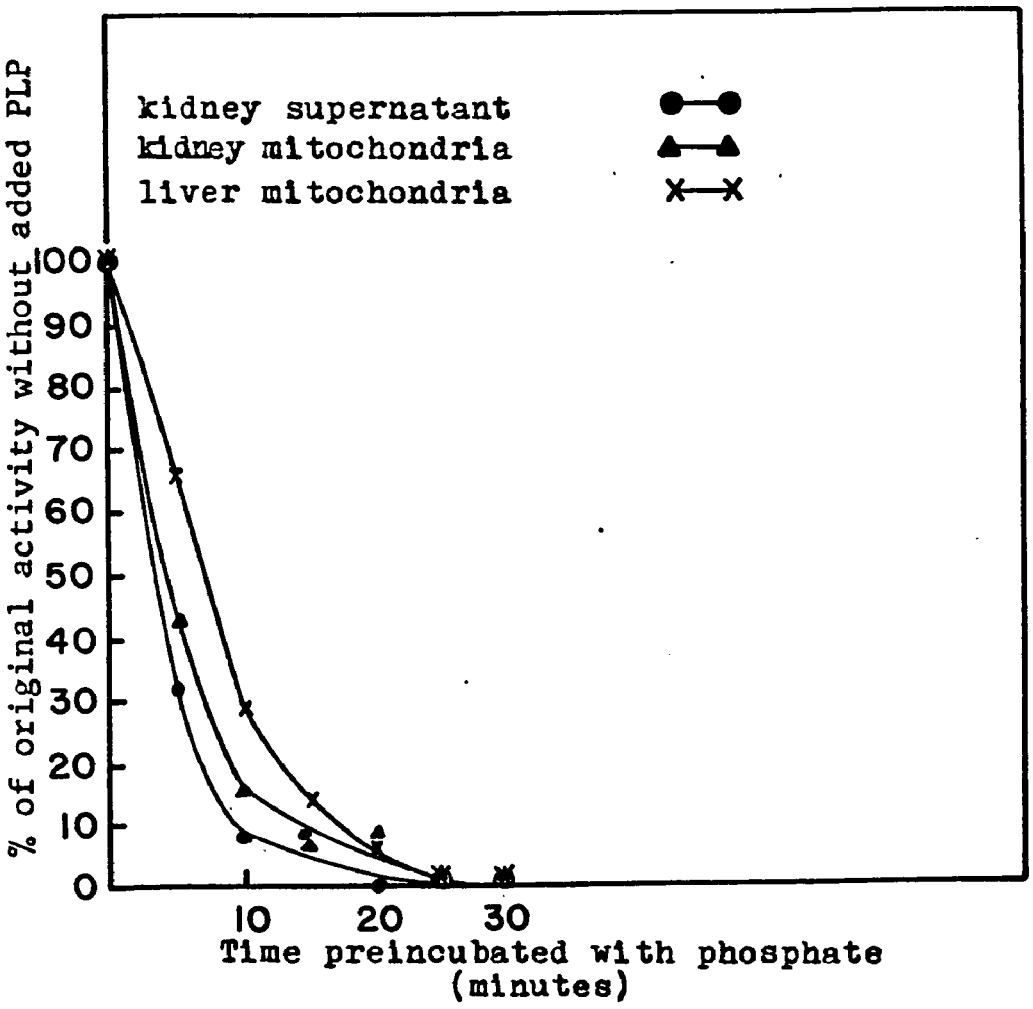
Diethylstilbestrol disulfate (SDS) inhibition The inhibition of KT by estrogen sulfates (see historical

Legend for Figure XI

Enzyme fractions were preincubated for various periods with 0.1 M phosphate buffer, pH 6.4, at 37°. They were then assayed in the presence and absence of pyridoxal phosphate. The activities presented in this figure are those obtained in the absence of pyridoxal phosphate additions. The activity obtained in the presence of added pyridoxal phosphate was 90 to 100 % of the original activity in the presence of added pyridoxal phosphate before preincubation with phosphate. Enough pyridoxal phosphate was added to achieve a concentration of 10^{-4} M.

Figure XI

Comparison of Phosphate Inhibition
of Kynurenine Aminotransferase Activities



section) was observed previously with kidney homogenates and purified preparations derived from kidney homogenates. We compared the effects of SDS on the reconstitution of KT activity of each of the three fractions by added PLP. Solubilization and resolution of the mitochondrial KTs were obtained by freezing and thawing. The apoenzyme fractions were incubated in the presence of 10^{-4} M PLP with and without SDS at various concentrations. The three KT preparations showed very similar sensitivities to SDS inhibition (Fig. XII).

Properties of Kynurenine Hydrolase

Comparison of the K_m values of kynurenine for kynurenine hydrolase and liver mitochondrial kynurenine aminotransferase We have pointed out in Part I that the potential activity level of the liver KT probably exceeds that of the KH *in vivo* but that the major pathway of kyn metabolism is via KH. One possible explanation for this anomaly is that KH has a lower K_m for kyn than does the liver KT *in vivo*. The K_m values of kyn for these two enzymes were compared both at their optimum pH values and at pH 7.4. The apparent K_m values are reported in Table XV and are derived from the reciprocal plots in Fig. XXI and XXII (Appendix). KH has a lower K_m for kyn than the liver KT at pH 7.4.

Legend for Figure XII

Previously frozen fractions were used in order to obtain solubilized apoenzyme preparations. The total incubation mixture (0.65 ml) contained 0.1 M Im buffer pH 6.5, 1 mM Ca^{++}, 4.6 mM αkg, 2.9 mM kynurenine SO_4, 0.1 mM PLP, 0.1 ml of enzyme fraction (equivalent to 20 mg wet weight of tissue), and various concentrations of SDS. Activity is reported as the % of the original activity assayed in the absence of any added SDS.

Figure XII

Comparison of the Degree of Inhibition of Various Kynurenine Aminotransferase Activities by Diethylstilbestrol Disulfate

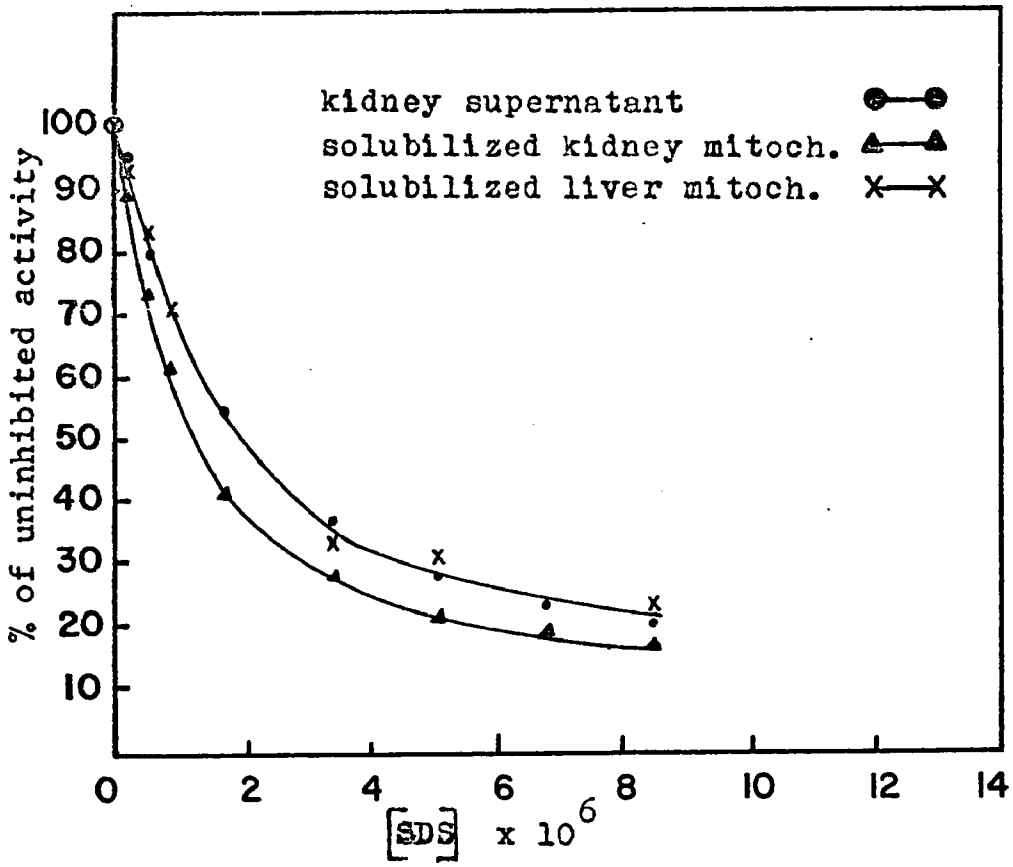


Table XV

Apparent K_m Values of Kynurenine for Crude Liver Mitochondrial Kynurenine Aminotransferase and Liver Kynurenine Hydrolase

pH	Kynurenine Aminotransferase	Kynurenine Hydrolase
6.5	3.2×10^{-3}	-
7.4	3.2×10^{-3}	1.7×10^{-4}
8.0	-	4.2×10^{-5}

Inhibition studies As mentioned in the historical section, KH appears to be rate limiting when kyn and 3OHkyn production rates are increased by TO induction or tryptophan administration. Although our results show that the KH is under hormonal control this does not negate the possibility of other types of control.

Moriguchi et al. (81) reported that the partially purified KH from Neurospora crassa was inactivated by incubation with L-alanine, a reaction product (Fig. IV). They presented evidence indicating that the KH catalyzes an enzymatic transamination of the PLP with the L-alanine. The reaction provides a possible mechanism of feed-back inhibition. This finding led us to test the effect of L-alanine on crude rat liver KH and 10-fold purified KH. We also tested other metabolites of kyn to see if any would inhibit the KH activity. The compounds tested were L-alanine,

pyruvate, α-ketoglutarate, α-ketoadipate, α-aminoadipate, kynurenic acid, xanthurenic acid, quinolinic acid, and nicotinamide. The concentrations used were approximately the same as that of the substrate, L-kynSO_4 (2×10^{-3} M). Assays were done in PP_i buffer, pH 8.1, with and without added PLP (10^{-4} M).

α-ketoglutarate was tested as a possible reverser of any transamination reaction that might have taken place in the tissues prior to or during homogenization. Pyruvate is a transamination product of alanine and thus was also a potential feedback inhibitor. The other compounds are metabolites that also occur in the metabolic pathway (Fig. II and III) and therefore might also exhibit some negative feedback control on the branch-point enzyme, KH. None of the compounds tested had any effect on the KH activity.

DISCUSSION

The results reported in this section verified the reliability of the KT and KH assay systems and provided several bases for the comparison of enzyme characteristics. Such comparisons allowed further evaluation of possible mechanisms by which estrogens regulate tryptophan metabolism.

During B_6-deficiency the kidney supernatant KT was depleted of PLP to a greater extent than either of the mitochondrial KT's (1,60). Depletion of PLP was accompanied by a loss of enzyme, i.e. a loss of ability to restore complete activity with added PLP. This difference might be explained by a greater affinity of the mitochondrial enzymes for PLP or by retention of PLP by the mitochondrial membrane. The different responses of the liver and kidney KT's to estrogens might also be explained by either different characteristics of the enzymes or different environments of the enzymes. Therefore, we compared several characteristics of the KT's.

The pH optima of the supernatant and mitochondrial KT's were similar (Fig. X). The apparent K_m values of substrates and cofactor for each enzyme did not reveal any gross differences (Tables XIII and XIV). These saturation data are interpreted as a rough index of the enzyme affinities for substrates and PLP on the assumption that large differences in affinity would be reflected in different K_m values. Our K_m values were in agreement with data presented previously for kyn and αkg for crude, solubilized rat liver

mitochondrial KT (82) and for PLP for purified rat kidney supernatant KT (1). The K_m value of PLP for the mitochondrial KT's would have to be lower than the K_m of PLP for the supernatant enzyme in order for one to conclude that the mitochondrial enzyme may have a greater affinity for PLP. Since the mitochondrial K_m was actually higher, the affinity for PLP appears not to be a factor in the response to B_6-deficiency. The KT's all responded similarly to phosphate (Fig. XI) and diethylstilbestrol (Fig. XII) inhibition. Thus, these data fail to indicate any differences in the catalytic properties of the KTs that might explain the different responses to B_6-deficiency or estrogen treatment. Other potential differences, i.e. differences in enzyme stability, were not explored.

Our experiments using αka indicate that it is a substrate for KT with a K_m value similar to that of αkg. (Tables XIII and XIV). This finding may be of regulatory importance for two reasons. αka is a product of the major pathway of tryptophan metabolism (Fig. III) and therefore may be in abundance especially during the catabolism of a tryptophan load. Secondly, αka exhibits a much greater substrate inhibition on KT activity (Appendix Fig. XXIII).

The apparent K_m values of kyn for KH and liver KT (Table XV) indicate that KH may have a greater affinity than KT for kyn at pH 7.4 or above. These data are relevant to the question posed in Part I as to why the major pathway of tryptophan metabolism proceeds via KH although liver KT

appears to have a higher potential activity level. If we assume that the K_m values adequately reflect conditions existing *in vivo* and that the *in vivo* pH is 7.4 or above, the much lower K_m value of KH could easily rationalize its preferential utilization of kyn. With similar assumptions, a cellular pH approaching 6.4 would negate this mode of control as the KH activity decreased. The preferential utilization via KH at these lower pH values might then occur as a result of the restricted access of the mitochondrial KT to substrate which occurs in this pH range but not at pH's above 7.0 (Mason, unpublished data).

Since our data do not reveal marked differences in the KT's, it becomes more likely that their different responses to B_6-deficiency and estrogen treatment are due at least in part to compartmentation. The level of PLP in the mitochondria was reported to be higher than in the cytosol during B_6-deficiency (83). This difference is consistent with the differences in percent saturation of the KT's with PLP (60). Even in animals that were not deficient in pyridoxine, we consistently observed a lower percent saturation of the supernatant enzyme, even though the mitochondrial fraction was exposed to twice as much diluent during fractionation as the cytosol. While these observations suggest retention of PLP by the mitochondrial membrane, there was no observable difference in the rate of inactivation of the supernatant and mitochondrial KT's by added inorganic phosphate. Since substrate anions are known to penetrate

the inner mitochondrial membrane by specific anion exchange reactions (84), it is possible that the inorganic phosphate exchanges with the mitochondrial PLP. The known degradative enzymes for PLP are in the cytosol, so the mitochondrial co-factor may be protected and may therefore be depleted less rapidly during B_6-deficiency. Further study of factors determining the penetration of PLP through the mitochondrial membrane should be useful in understanding the distribution and function of B_6-vitamers.

Various types of feedback control have been described for metabolic systems in bacteria. These mechanisms sometimes involve products of alternate paths of metabolism interacting with branch-point enzymes to selectively inhibit further product formation. It has already been shown that alanine can inhibit KH from Neurospora, so we tested various compounds involved in tryptophan metabolism for possible feedback control of KH. We were unable to find evidence for such inhibition of the rat liver KH by alanine or any of the various other metabolites.

SUMMARY

We have investigated factors of potential regulatory importance in the synthesis of kynurenine by tryptophan oxygenase and in its degradation by kynurenine hydrolase and kynurenine aminotransferase. Factors which affect the activities of these enzymes *in vitro* and their apparent tissue levels *in vivo* were studied.

A. In comparisons of the characteristics of the rat liver mitochondrial aminotransferase and the supernatant and mitochondrial kidney aminotransferase fractions it was observed that:

1. their pH optima were very similar.
2. their apparent K_m values for kynurenine and α-ketoglutarate were similar. Small differences in their apparent K_m's for pyridoxal phosphate were noted.
3. α-ketoadipate, a product of the further catabolism of kynurenine, was a substrate and had apparent K_m values for the three aminotransferases similar to those of α-ketoglutarate.
4. their rate and degree of inactivation in 0.05 M phosphate buffer were similar.
5. their degree of inhibition by various concentrations of diethylstilbestrol disulfate was very similar.

In studies of the characteristics of the kynurenine hydrolase preparations, it was observed that:

1. potential feedback inhibitors, including alanine, pyruvate, α-ketoadipate, α-aminoadipate, kynurenic

acid, xanthurenic acid, quinolinic acid, and nicotinamide, were not inhibitory at concentrations equimolar with the knynurenine concentration.

2. the apparent K_m for the substrate, kynurenine, was 4.2×10^{-5} at pH 8.0 and 1.7×10^{-4} at pH 7.4.

B. In studies of the apparent tissue levels of tryptophan oxygenase, kynurenine hydrolase, and the three kynurenine aminotransferases, it was observed that:

1. the diurnal variation of tryptophan oxygenase activity was not accompanied by significant variation of the apparent levels of kynurenine hydrolase, or of the three aminotransferases.
2. subcutaneous administration of estradiol benzoate (10 µg per day for 16 to 30 days) to adult female rats caused a two-fold increase in tryptophan oxygenase activity.
3. adult male and female rats did not exhibit a sex difference in tryptophan oxygenase activity levels.
4. oral administration of 17 β-estradiol (15 µg to 20 µg per day for 7 to 40 days) to male rats caused the kidney kynurenine aminotransferase activities to remain at low levels similar to the female levels.
5. subcutaneous administration of estradiol benzoate (10 µg per day for 16 to 30 days) to male rats caused kynurenine hydrolase and kidney kynurenine aminotransferase activities to remain at low levels similar to the female levels.

6. L-tryptophan administered orally (200 mg per day for 1 to 37 days) or by intraperitoneal injection (25 mg per day per 100 g body weight for 7 to 30 days) to male and female rats had no effect on kynurenine hydrolase and kynurenine aminotransferase activity levels.
7. oral administration of cortisol-21-phosphate (3 to 4 mg daily for 5 to 26 days) to male rats did not cause any significant changes in the kidney kynurenine aminotransferase activities and resulted in an increase in the liver kynurenine aminotransferase and kynurenine hydrolase activity levels. Lower dosages gave similar results (0.3 mg to 2 mg daily for 5 to 26 days).
8. intraperitoneal pyridoxine administration (1 to 3 mg per day for 9 to 16 days) had no significant effect on activity levels or the per cent saturation with pyridoxal phosphate of kynurenine hydrolase and the three kynurenine aminotransferases.
9. castration of male rats resulted in a decrease in kynurenine hydrolase and kidney mitochondrial kynurenine aminotransferase activities toward female levels.
10. intramuscular injections of testosterone propionate (1 mg per day for 5 to 18 days) to female rats caused an increase in kidney kynurenine aminotransferase activities toward the male levels.

C. These results have clarified several unanswered questions concerning the regulation of tryptophan metabolism:

1. Why does the pathway proceed via kynurenine hydrolase predominantly rather than via kynurenine aminotransferase? Our results indicate an excess of potential kynurenine aminotransferase activity over potential kynurenine hydrolase activity, so the potential kynurenine aminotransferase activity is not expressed fully in vivo. This inactivity may be explained by the much lower K_m of kynurenine for kynurenine hydrolase than for kynurenine aminotransferase or by the intramitochondrial location of most of the kynurenine aminotransferase. Since the K_m's and the penetration of kynurenine aminotransferase substrates through the mitochondrial membrane are pH dependent, the relative roles of each factor cannot be assessed from available data.
2. Why do exogenous estrogens cause a diversion of tryptophan metabolism to the kynurenine aminotransferase pathway? We have assumed that they do so by stimulating kynurenine accumulation either by decreasing kynurenine hydrolase activity or increasing tryptophan oxygenase activity. Our results which confirm that both actions result from estradiol benzoate injections support this suggestion.
3. Why does supplemental pyridoxine correct the estrogen effect mentioned in (2)? It has been

assumed that such supplements prevent kynurenine accumulation by accelerating kynurenine hydrolase activity, presumably by increasing the saturation of the hydrolase with pyridoxal phosphate. Our *in vitro* results do indicate incomplete saturation (about 60%) of the hydrolase with pyridoxal phosphate. However, we were unable to decrease that value either by estrogen treatment or increase it by pyridoxine administration. The validity of the assumption remains to be established.

4. *Is there a sex difference in tryptophan oxygenase activity levels?* We have been unable to detect one. The lack of one would indicate that the above-mentioned elevation of tryptophan oxygenase activity by exogenous estrogen may not have a physiological counterpart. On the other hand, the effect of exogenous estrogen in decreasing kynurenine hydrolase activities may provide a tentative explanation for the lower kynurenine hydrolase activity seen in female rats.

5. *Are the effects of estrogens on kynurenine hydrolase and kynurenine aminotransferase activities mediated through their stimulation of corticosteroid accumulation?* The results of our studies of the effects of estrogens and corticosteroids on the tryptophan oxygenase, kynurenine aminotransferase, and kynurenine hydrolase activities demonstrate that

these hormones have completely different effects on the enzyme activities. The estrogen treatment studies have shown that kynurenine hydrolase and kynurenine aminotransferase levels can be decreased without any effect on the tryptophan oxygenase levels indicating that these treatments were not accompanied by increases in corticosteroid levels sufficient to induce tryptophan oxygenase. The diurnal variation studies and studies of the effects of tryptophan or corticosteroid administration have shown that the oxygenase levels can be increased without any decrease in the hydrolase or aminotransferase levels. It appears, therefore, that the estrogen effects on kynurenine aminotransferase and kynurenine hydrolase activities are independent of their ability to stimulate corticosteroid accumulation.

6. How are the effects of estrogens on kidney kynurenine aminotransferase levels related to the observed sex differences? It was previously reported that treatment of male rats with diethylstilbestrol caused them to exhibit depressed kynurenine aminotransferase activities similar to the female levels. This study has shown for the first time that exogenous 17 β-estradiol, a natural estrogen, has a similar action. It has also shown that castration decreases the male aminotransferase levels and exogenous testosterone propionate increases the female levels. It is

possible that the estrogens depress kynurenine aminotransferase activities by inhibiting testosterone production or that testosterone may inhibit the production of estrogens or its action. The former possibility is consistent with the known trophic effects of testosterone on the kidney. Increased enzyme production is typical of the known effects of steroid hormones, whereas the apparent depression of kynurenine aminotransferase production exhibitied by estrogens is atypical. The sex difference probably reflects the relative levels of androgens and estrogens in the two sexes.

7. What role does the availability of pyridoxal phosphate have in controlling the activities of kynurenine hydrolase and kynurenine aminotransferase? We were unable to significantly increase the apparent kynurenine hydrolase or kynurenine aminotransferase levels in female rats or their per cent saturation by supplemental pyridoxine. This result indicates that the low enzyme levels in females are not a result of a relative lack of pyridoxal phosphate. The decline in the apparent levels of these two enzymes during pyridoxine deficiency and the lower per cent saturation with pyridoxal phosphate seen in males and in testosterone-treated females remain to be explained.

8. Why is the per cent saturation of the supernatant kynurenine aminotransferase more rapidly decreased than that of the mitochondrial kynurenine aminotransferase during pyridoxine deficiency? We found no indication in comparing the K_m's of kynurenine aminotransferases for pyridoxal phosphate or their substrates that the binding of pyridoxal phosphate to the mitochondrial enzyme is greater than to the supernatant enzyme. It is more likely, therefore, that the mitochondrial pyridoxal phosphate persists because it is protected from the degradative enzymes located in the cytosol.

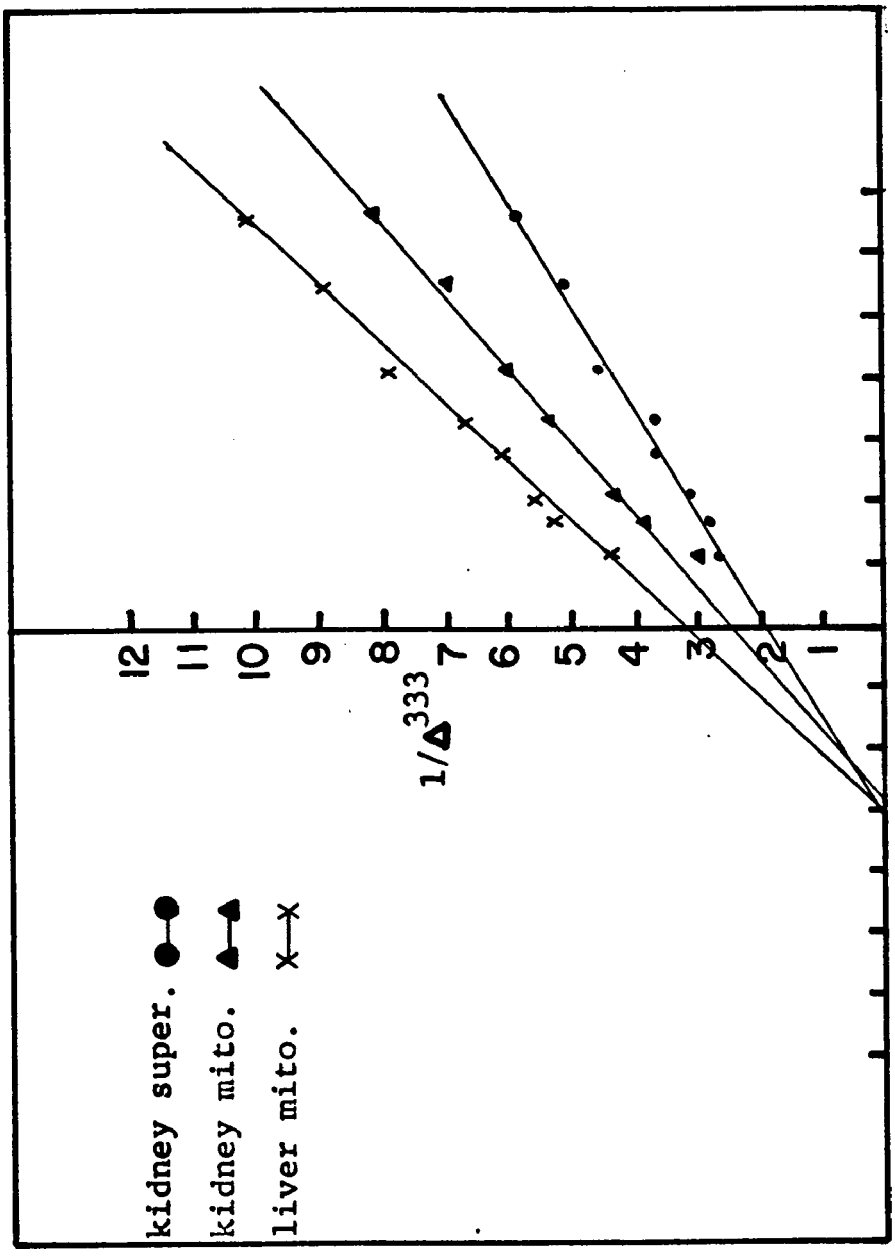
APPENDIX

Legend for Figure XIII

Identical conditions were used for the several fractions. The total incubation volume was 0.62 ml. It contained 0.1 M Im buffer, pH 6.5, 0.48 mM $CaCl_2$, 0.97 mM PLP, 1.9 mM αkg, and 0.05 ml of kidney mitochondrial or supernatant fraction (equivalent to 10 mg wet weight of tissue) or 0.1 ml of liver mitochondrial fraction (equivalent to 20 mg wet weight of tissue). The incubations were for 30 minutes at 37°. Δ_{333} is the corrected O.D., which is proportional to the amount of kynurenic acid present (see Methods).

Figure XIII

Reciprocal Plots for Kynurenine Sulfate Saturation Data for Crude Kynurenine Aminotransferases



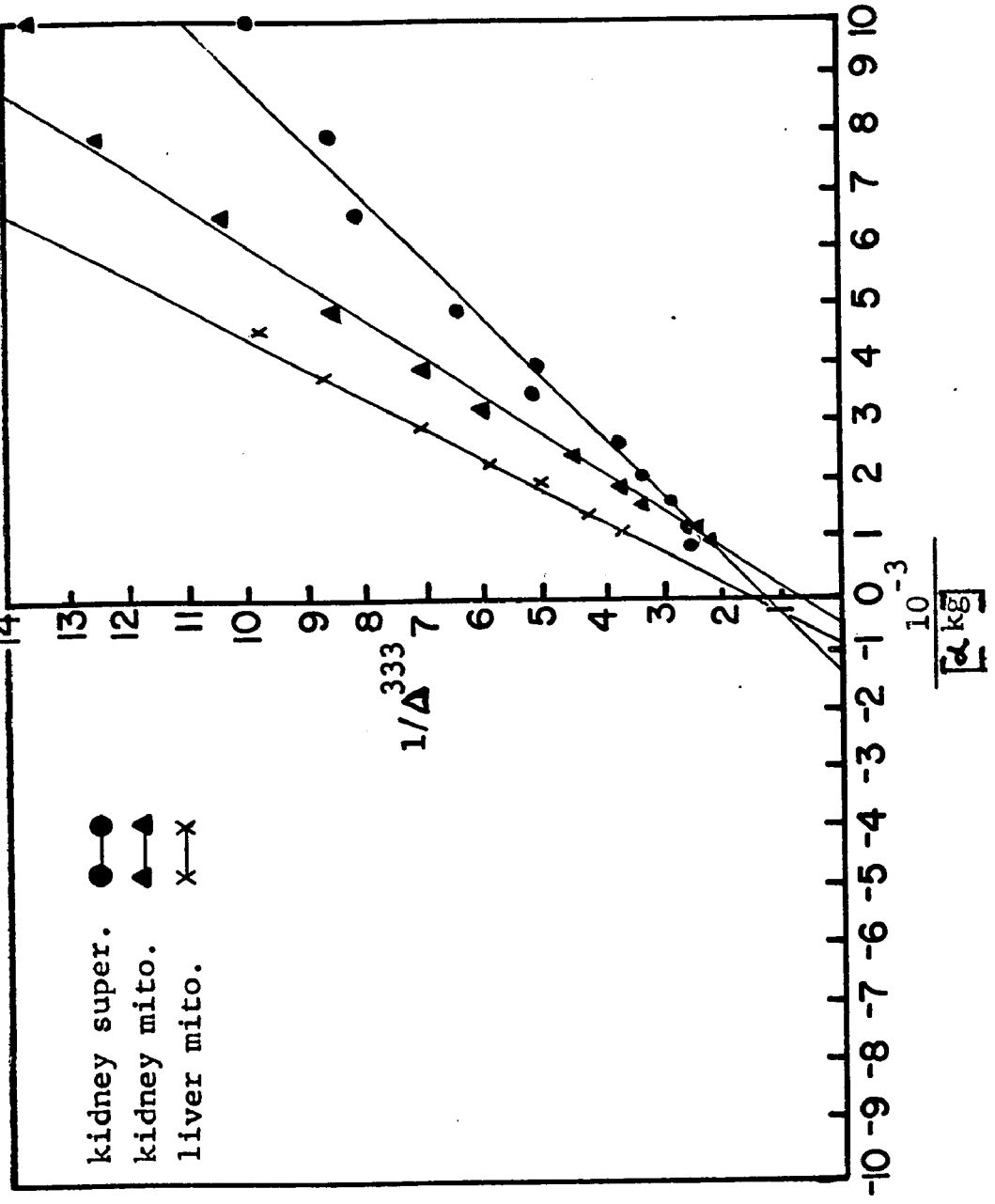
Legend for Figure XIV

Fractions were prepared in 0.25 M sucrose as described in Methods. The total incubation volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 0.1 mM PLP, 3.1 mM $kynSO_4$, and 0.1 ml of kidney supernatant or mitochondrial fraction (equivalent to 20 mg wet weight of tissue) or 0.2 ml of liver mitochondrial fraction (equivalent to 40 mg wet weight of tissue). Incubations were for 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present (see Methods).

Figure XIV

Reciprocal Plots for α-ketoglutarate Saturation

Data for Crude Kynurenine Aminotransferases

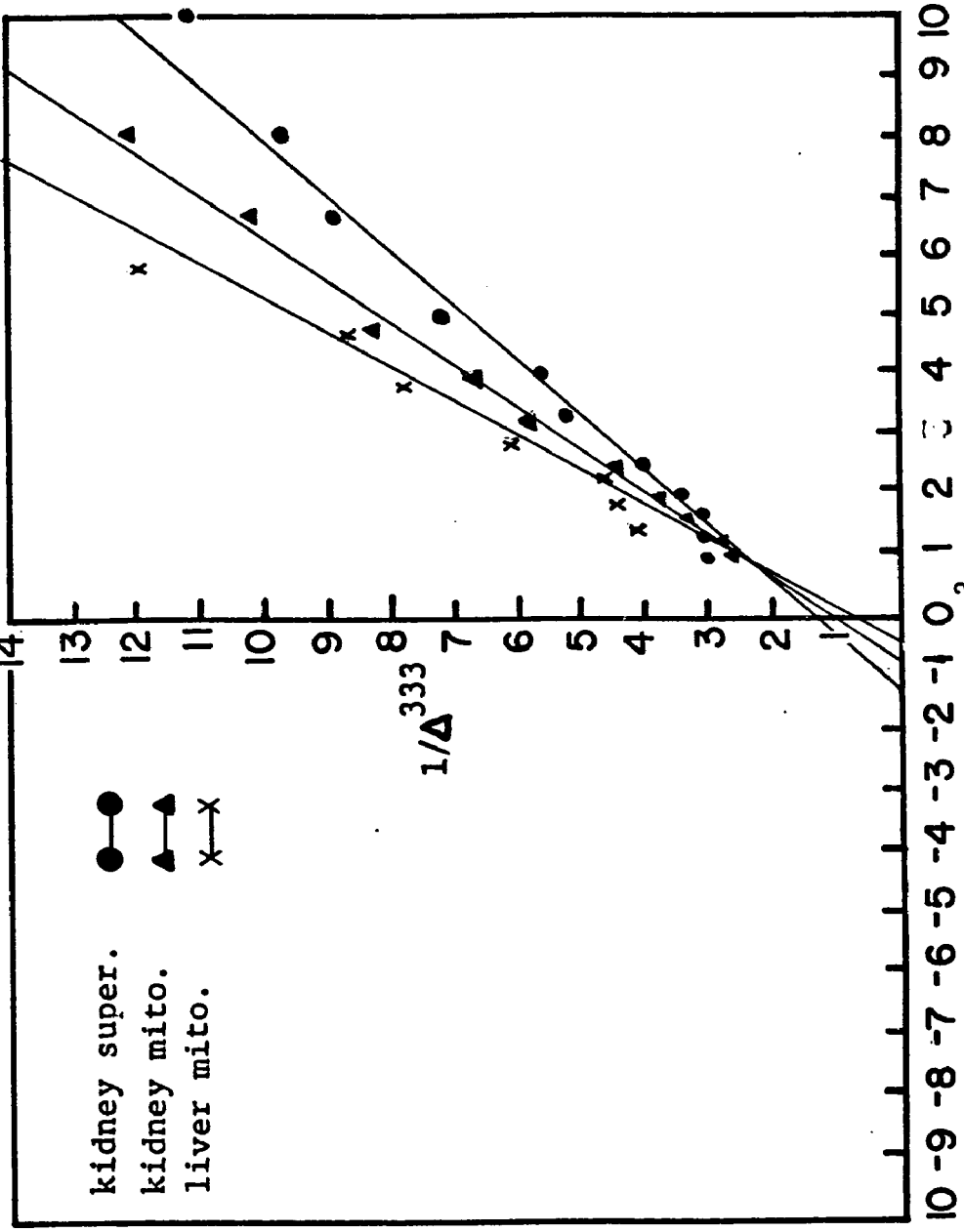


Legend for Figure XV

Fractions were prepared in 0.25 M sucrose as described in Methods. The total incubation volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 0.1 mM PLP, 3.1 mM $kynSO_4$, and 0.1 ml of kidney supernatant or mitochondrial fraction (equivalent to 20 mg wet weight of tissue) or 0.2 ml of liver mitochondrial fraction (equivalent to 40 mg wet weight of tissue). Incubations were for 30 minutes at 37°. $\Delta 333=$ corrected O.D., which is proportional to the amount of kynurenic acid present (see Methods).

Figure XV

Reciprocal Plots for α-ketoadipate Saturation Data for Crude Kynurenine Aminotransferases

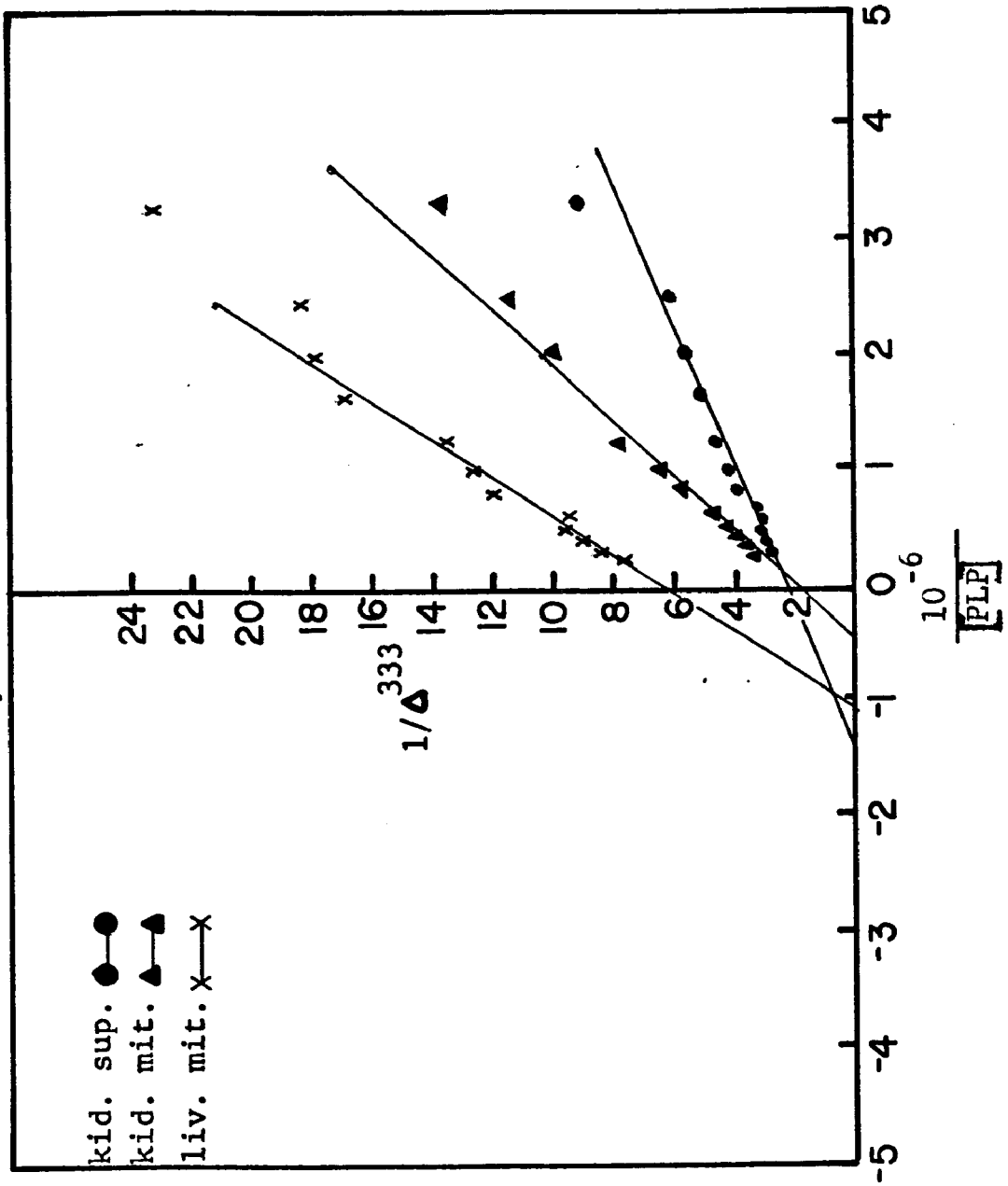


Legend for Figure XVI

The total incubation mixture volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 5 mM, αkg, 3.1 mM kynSO4, and 0.1 ml of each enzyme fraction (equivalent to 20 mg wet weight of tissue). All enzyme fractions were preincubated at room temperature for 15 minutes with buffer, αkg, and PLP to allow holoenzyme formation. Then kyn was added and incubations were carried out for 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present (see Methods).

Figure XVI

Reciprocal Plots for Pyridoxal-5-phosphate Saturation Data for Crude Kynurenine Aminotransferases

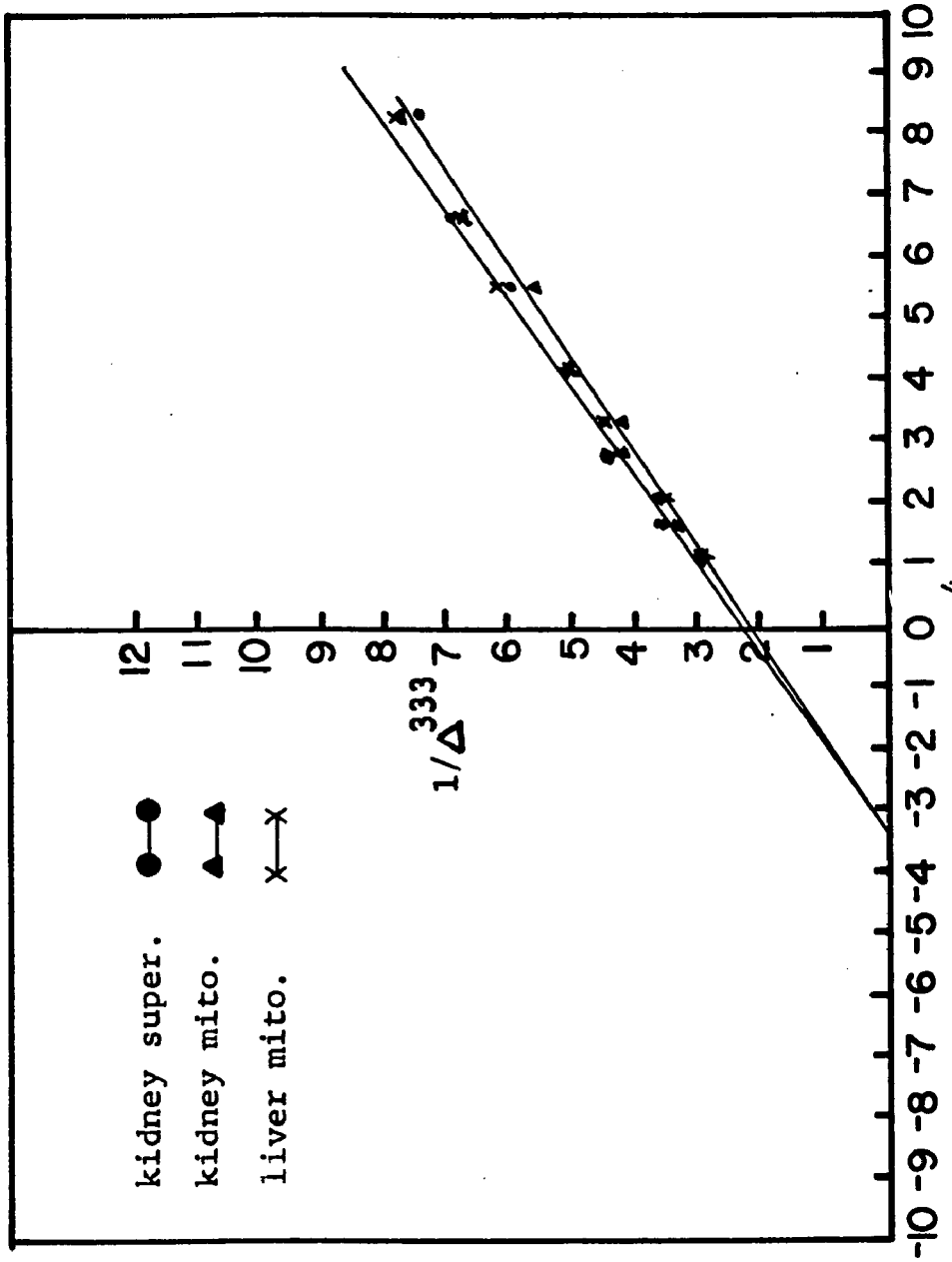


Legend for Figure XVII

The total incubation volume was 0.62 ml. It contained 0.1 M Im buffer, pH 6.5, 0.48 mM $CaCl_2$, 0.097 mM PLP, 1.9 mM αkg, and 0.05 ml of enzyme fraction (equivalent to 0.14 mg of protein in the kidney supernatant fraction, 0.06 mg of protein in the kidney mitochondrial fraction, and 0.57 mg of protein in the liver mitochondrial fraction). Assays were for 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XVII

Reciprocal Plots for Kynurenine Sulfate Saturation Data for Partially Purified Kynurenine Aminotransferases

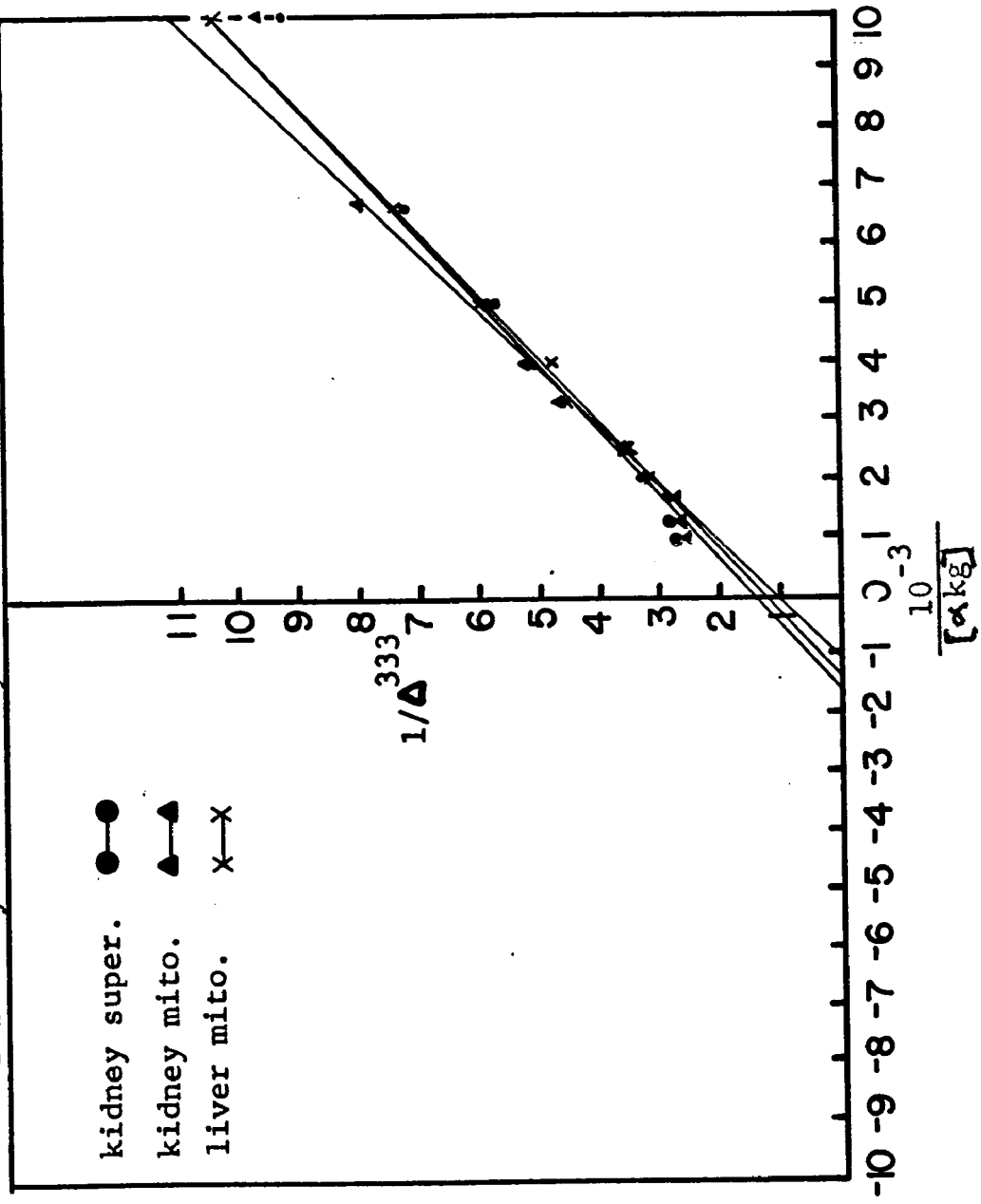


Legend for Figure XVIII

The total incubation volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 0.1 mM PLP, 3.1 mM $kynSO_4$, and 0.1 ml of enzyme fraction (equivalent to 0.28, 0.11, and 1.15 mg of protein for the kidney supernatant, kidney mitochondrial, and liver mitochondrial fractions respectively). The incubation period was 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XVIII

Reciprocal Plots for α-ketoglutarate Saturation Data for Partially Purified Kynurenine Aminotransferases

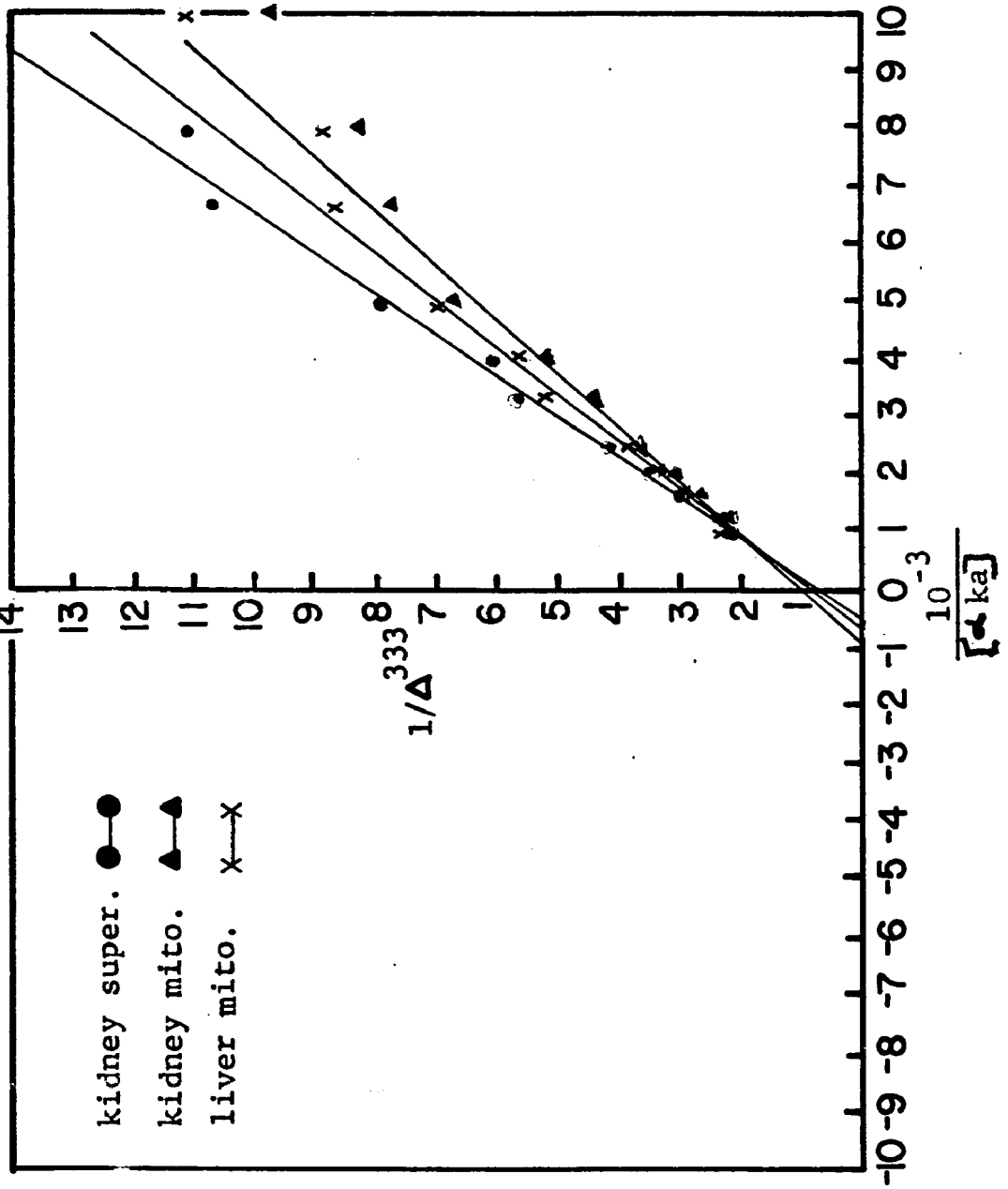


Legend for Figure XIX

The total incubation volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 0.1 mM PLP, 3.1 mM $kynSO_4$, and 0.1 ml of enzyme fraction (equivalent to 0.28, 0.11, and 1.15 mg of protein for the kidney supernatant, kidney mitochondrial, and liver mitochondrial fractions respectively). The incubation period was 30 minutes at 37^0. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XIX

Reciprocal Plots for α-ketoadipate Saturation Data for Partially Purified Kynurenine Aminotransferases

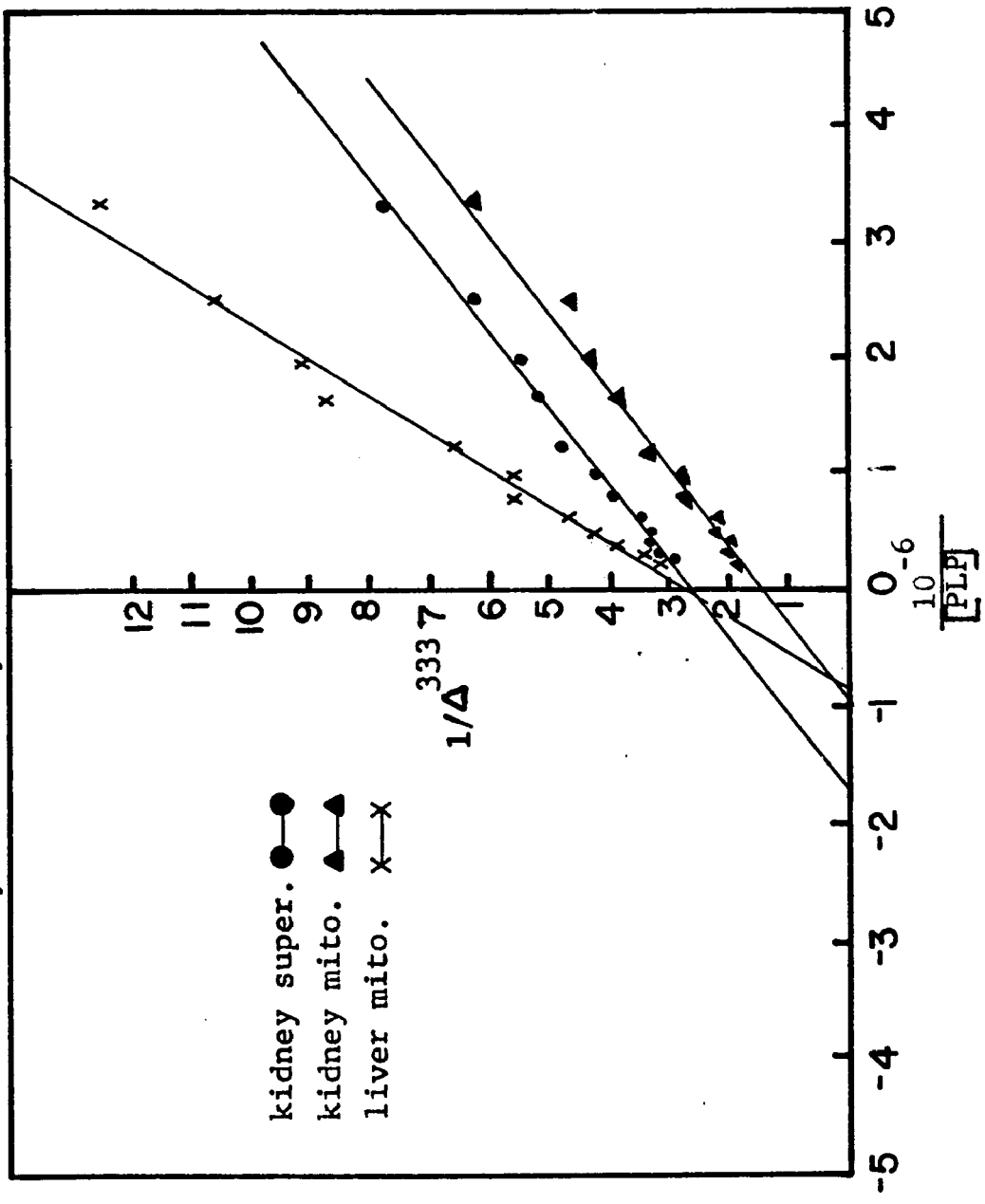


Legend for Figure XX

The total incubation volume was 0.6 ml. It contained 0.1 M Im buffer, pH 6.5, 0.5 mM $CaCl_2$, 5 mM αkg, 3.1 mM $kynSO_4$, and 0.1 ml of enzyme fraction (equivalent to 0.56, 0.11, and 2.3 mg of protein for the kidney supernatant, kidney mitochondrial, and liver supernatant fractions respectively). All enzyme fractions were preincubated at room temperature for 15 minutes with buffer, αkg, and PLP to allow holoenzyme formation. Then kyn was added and incubations were carried out for 30 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XX

Reciprocal Plots for Pyridoxal-5-phosphate Saturation Data for Partially Purified Kynurenine Aminotransferases

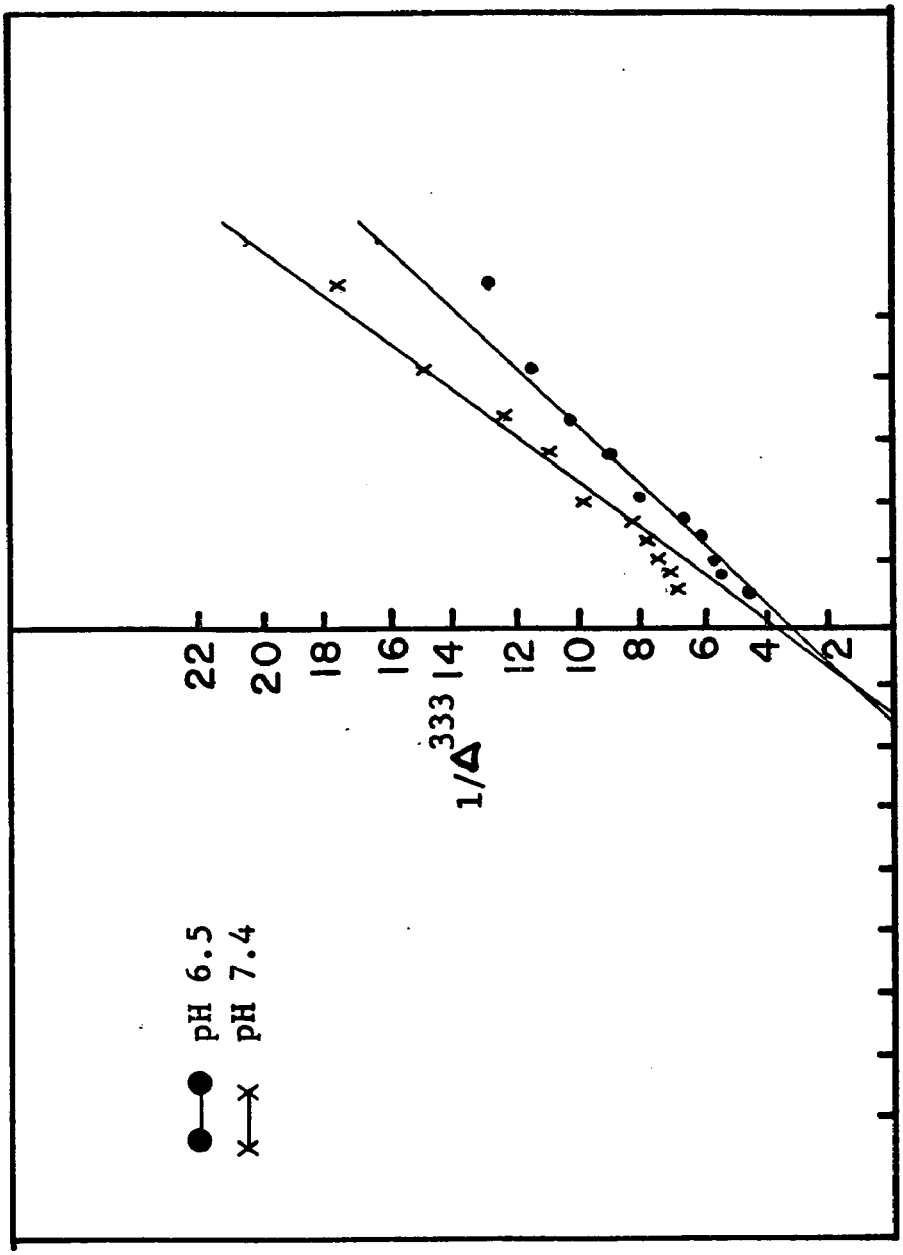


Legend for Figure XXI

The total incubation volume was 0.62 ml. It contained 0.1 M Im buffer, pH 6.5, or 0.05 M P_i buffer, pH 7.4, 0.48 mM $CaCl_2$, 0.97 mM PLP, 1.9 mM αkg, and 0.1 ml of liver mitochondrial fraction (equivalent to 20 mg wet weight of tissue). Incubations were for 30 minutes at 37^0. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XXI

Reciprocal Plots of Saturation Data for Kynurenine Sulfate for Liver Mitochondrial Kynurenine Aminotransferase at pH 6.5 and 7.4

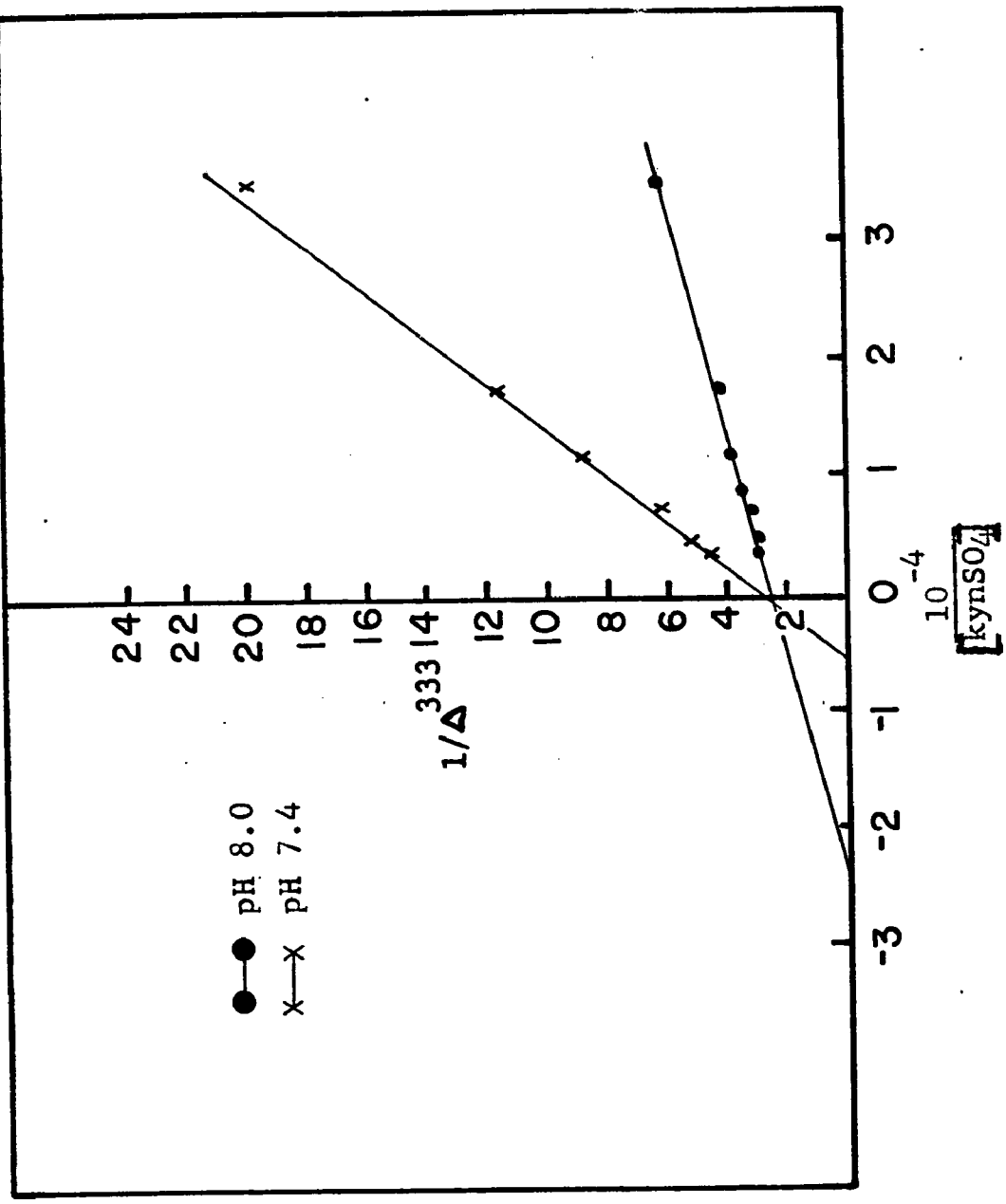


Legend for Figure XXII

The total incubation volume was 1.3 ml. It contained 0.05 M PP_i buffer, pH 8.0, or 0.05 M P_i buffer, pH 7.4, 0.09 mM PLP, and 0.4 ml of enzyme fraction (equivalent to 80 mg wet weight of tissue). Incubations were for 30 minutes at 37°. Enzyme activity is expressed as OD_{560}, which is proportional to the amount of anthranilic acid present.

Figure XXII

Reciprocal Plots of Saturation Data for Kynurenine Sulfate for Kynurenine Hydrolase at pH 7.4 and 8.0

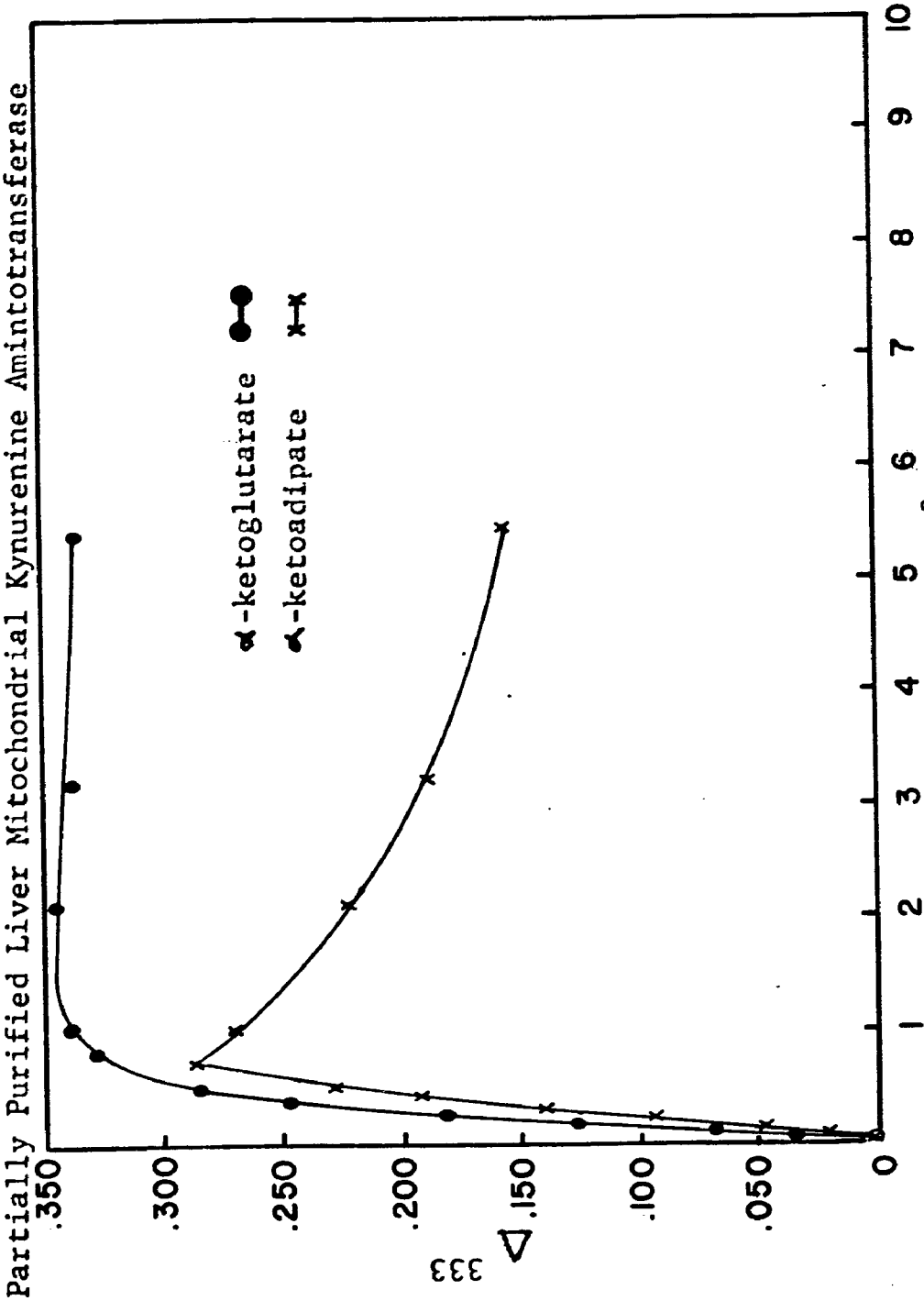


Legend for Figure XXIII

Fractions were partially purified as described in the text. Incubation mixtures contained 0.05 M P_i buffer, pH 6.4, 0.18 mM PLP, 3 mM $kynSO_4$, and 0.1 ml of enzyme fraction (equivalent to 4 mg protein). Incubations were carried out for 15 minutes at 37°. Δ^{333} = corrected O.D., which is proportional to the amount of kynurenic acid present.

Figure XXIII

Saturation Data of ∝-ketoglutarate and ∝-ketoadipate for Partially Purified Liver Mitochondrial Kynurenine Amintotransferase



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