I

ALTERNATE PATHWAYS OF TRYPTOPHAN METABOLISM IN THE RAT

BY

Edith E. Townsend

THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

ALLAN MEMORIAL INSTITUTE OF PSYCHIATRY

MONTREAL, QUEBEC. April 20, 1959.

ACKNOWLEDGEMENTS

This investigation was aided by a grant to Dr. T.L. Sourkes from the Federal-Provincial Mental Health Fund and by a grant from the Foundations Fund for Research in Psychiatry, New Haven, to Dr. R.A. Cleghorn.

I would like to express my sincere thanks and appreciation to Dr. T.L. Sourkes for his valuable guidance, assistance and encouragement throughout this investigation. I am grateful to Dr. Sourkes for performing one of the statistical analyses reported in this thesis.

I am indebted to Dr. R.A. Cleghorn for his constant interest in and encouragement of this research. I am also grateful to Dr. Cleghorn for assistance in performing many of the adrenalectomies where they were necessary.

I am grateful to J. Katis for assistance in one experiment involved in determining the effect of dietary tryptophan on 5-hydroxyindoleacetic acid excretion.

I would like to thank Miss Gail MacArthur for her excellent job in typing this thesis and members of the department for their helpful suggestions and constructive criticisms in the preparation of this manuscript.

ABBREVIATIONS

5-HT	5-hydroxytryptoptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HTP	5-hydroxytryptophan
TP	tryptophan pyrrolase
B_1	thiamine
DPT	diphosphothiamine (cocarboxylase)
PLP	pyridoxal phosphate

TABLE OF CONTENTS

page

page

ERRATUM

P. 9, Line 15. This sentence should read: "This enzyme requires pyridoxal phosphate as coenzyme and the apoenzyme is increased in activity by the administration of hydrocortisone to the animal (36)."

P. 110, Line 23. This sentence should read: "Two factors are known which influence the metabolism of a tryptophan load: 1) the adaptive increase in TP activity thereby facilitating degradation of tryptophan via the route forming kynurenine and 2) the relatively large capacity of the tryptophan-α-ketogluarate transaminase system to degrade tryptophan via indolepyruvic acid."

P. 112, Line 2. This sentence should read: "1) TP activity in the liver is increased by the administration of a tryptophan load;..."

P. 119, Line 9. Delete sentence "It must be kept in mind...."

Line 11. This sentence should read: "Tryptophan-α-ketoglutarate transaminase may account for as much of the metabolism of tryptophan as the tryptophan pyrrolase system (157)."

I INTRODUCTION

A. PURPOSE OF THIS RESEARCH

The work to be presented in this thesis concerns the divergent pathways of tryptophan metabolism to the 5-hydroxyindoles and to kynurenine. More specifically an attempt has been made to elucidate the role of dietary tryptophan in the operation of these two pathways.

Previous investigators have implied that thiamine is involved in the kynurenine-forming pathway. Both nutritional studies and experiments *in vitro* were carried out to determine just where thiamine (or thiamine pyrophosphate, its coenzyme form) exerts its action. In order to elucidate the enzymatic influence of thiamine, experiments *in vitro* on the system forming L-kynurenine from L-tryptophan and on kynureninase were performed.

In the course of this investigation attempts were made to relate the activity of the adaptive system forming kynurenine, in effect, the activity of tryptophan pyrrolase (tryptophan peroxidase-oxidase), to the dietary content of tryptophan. Nutritional deficiency studies have helped to clarify this relationship.

It was of further interest to establish the effects of various derivatives of tryptophan on the 'adaptability' of tryptophan pyrrolase *in vitro*.

Nutritional studies were carried out to determine the effect of tryptophan deficiency on the formation of 5-hydroxyindoles, and the turnover of 5-hydroxyindoles *in vivo*.

B. DISCOVERY AND IDENTIFICATION OF TRYPTOPHAN

The initial studies of tryptophan resulted from the early observation by Adamkiewicz (1) in 1874 of the violet colour which appears when a mixture of glacial acetic acid and albumin is treated with sulphuric acid. Hopkins and Cole (2) later found that colour production requires an impurity in the glacial acetic acid, glyoxylic acid, and pondered on the substance in the "proteids" which reacts with the glyoxylic acid. These investigators were able to isolate crystals from the pancreatic digestion of casein, crystalline egg albumin and hydrolysed serum "proteids" which gave a positive 'glyoxylic reaction' and a red-rose colour with bromine water (3). Tryptophan (from the Greek, phanein - to appear) was obtained from the tryptic digestion of protein. Hopkins and Cole found that tryptophan yields indole and skatole on heating and that the putrefactive action of bacteria produces skatole, skatole-acetic and skatole-carboxylic acids from tryptophan. The putrefaction of gelatin did not yield indoles and no glyoxylic acid reaction was obtained. These investigators concluded that tryptophan is not a component of gelatin (4). They also established the correct empirical formula - $C_{11}H_{12}N_2O_2$. Ellinger and Flamand, in 1907 (5), synthesized tryptophan, α-amino-β-3-indolepropionic acid, from 3-indolealdehyde and hippuric acid, thus clearing up the error of Hopkins and Cole who thought tryptophan was skatolyl-2-aminoacetic acid. Tryptophan has since been found in many proteins but has not been found as a major constituent.

C. NUTRITIONAL REQUIREMENT FOR TRYPTOPHAN

As early as 1906 Willcock and Hopkins observed that zein as the sole nitrogenous constituent of the diet does not support the growth of mice. Although

the addition of tryptophan does not maintain growth, it does prolong the survival period and improves the well-being of the animals (6). Mendel compared various proteins for their ability to support the growth of young rats. He found that casein supports growth excellently but zein does not, unless the diet is supplemented with lysine and tryptophan (7). The nutritive evaluation of certain proteins led Osborne and Mendel in 1914 (8) to recognize the essential nature of some of the amino acids which their purified substances contain. In this way the fact was established that certain amino acids can be synthesized in animal tissues while others cannot. Osborne and Mendel clearly demonstrated that tryptophan and lysine are indispensable components of the diet. Rose, after studies in which the dietary protein was replaced by mixtures of purified amino acids, was able to summarize the minimum requirement of the ten amino acids essential for the promotion of growth of the rat (9). These amino acids are lysine, tryptophan, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, valine and arginine. The tryptophan requirement was established at 0.2% of the diet. These ten amino acids were found to be indispensable in the sense that the rat is unable to synthesize them; however, from these ten the rat is able to synthesize at least twelve other amino acids. In 1948, Rose, Oesterling and Womack noted that the rate of gain of rats fed diets containing ninteen amino acids was superior to that of rats whose diets contained only the ten essential amino acids but that growth of the latter group could be stimulated by the addition of glutamic acid (10). It can be inferred then that the non-essential amino acids complement the essential amino acids in their multiform biological roles. Williams et al. estimate the nutritional

requirement for tryptophan by carcass analysis to be 0.1%. They attribute the discrepancy between this value and the nutritionally determined one to a possible deficiency of niacin in the diets used in the latter studies (11). On the other hand the carcass analysis may not necessarily account for all the tryptophan-derived carbon.

Rose, Berg and their associates, as well as other groups, have tested many tryptophan derivatives for their ability to replace tryptophan in the diet of growing rats. Both the D- and L-isomers of tryptophan were found to promote growth in the rat (12,13,14). Acetyl-tryptophan is utilized (15) but only in the L-form (13,14). It was postulated that deacetylation occurs after absorption from the intestine (15). Tryptophan ethyl ester hydrochloride is also efficiently utilized, probably after hydrolysis in the alimentary tract (15). N-Methyltryptophan (abrine) is an effective replacement for tryptophan in the diets of growing rats (16,17) whereas the methyl derivatives of tryptophan substituted in the 2'- and 5'-positions of the indole component are not (16). Methylene and benzoyl tryptophan do not replace the free tryptophan (15). 3-Indolepyruvic acid (18) and DL-β-3-indolelactic acid (19) were found to be remarkably efficient in replacing the tryptophan supplement required for the growth of rats on diets containing acid-hydrolysed casein. 3-Indolepropionic acid (18), β-3-indoleacrylic acid and α-oximino-β-3-indolepropionic acid (19) are not able to replace tryptophan. Berg and Baugess studied the rate of absorption of tryptophan and tryptophan derivatives from the gastrointestinal tract of the rat. They found the rates of absorption to be as follows, in descending order: acetyl-DL-tryptophan, acetyl-L-tryptophan, L-tryptophan, DL-tryptophan, DL-tryptophan ethyl ester,

and L-tryptophan ethyl ester (20).

In addition to the loss of weight and negative nitrogen balance which results from tryptophan deficiency other symptoms have been recorded. Totter and Day (21) found in young rats some nervous manifestation, hyperexcitability, hunchback, unkempt appearance, alopecia, and greasy hair. In a large proportion they found vascularization of the cornea and, in a few, a generalized ophthalmia. Albanese and Buschke (22) found that cataracts develop only in growing animals and that the defects are of two kinds, chronic and acute. The former mature within a week preceding the death of the animals. In addition to the previously mentioned manifestations, they found defects of the incisor teeth, and atrophy of the testis and aspermiogenesis in males. Albanese and Randall (23) found that female rats administered tryptophan deficient diets after mating fail to cast a litter and that resorption of the embryos occurs.

Single amino acid deficiencies are rare under "natural" or clinical conditions, because dietary requirements are usually met by a mixed protein diet. However, pellagra is an example of a disease arising in part from a diet low in tryptophan. Goldberger and his associates in 1915 (24) were the first to recognize that pellagra is due to a nutritional deficiency. In a dietary experiment on institutional inmates they found that by increasing the animal and leguminous protein foods and reducing the carbohydrate food a remarkable recuperative or preventative effect was achieved. In 1922, Goldberger and Tanner reported that an amino acid deficiency is probably the primary etiological factor in pellagra. The administration of tryptophan and

cystine to pellagrins was found to provide a steady gain in weight and alleviation of other symptoms (25). Diets high in their content of corn have long been etiologically associated with pellagra, a fact which may be correlated with the low content of tryptophan in the edible portion. As will be seen later, dietary tryptophan is converted in part to nicotinic acid and in marginal diets the amount of vitamin available from this source may be critical for the health of the individual.

D. INTERMEDIARY PATHWAYS OF TRYPTOPHAN METABOLISM

i) General Outline

The multiform pathways of tryptophan metabolism are represented in the diagram of Fig. 1. For the sake of simplicity conjugated urinary products have been omitted. The pathways are a) incorporation into tissue protein, b) the 5-hydroxyindole pathway, c) the decarboxylative pathway yielding tryptamine, d) the transaminative pathway yielding indolepyruvic acid and e) the kynurenine-yielding pathway with its multiplicity of intermediary routes. The extent to which each of these pathways is normally operative is only now becoming known, as methods for determining minor metabolites are worked out. It must be kept in mind (a) that probably not all the possible intermediates and derivatives have been determined, and (b) that a valid estimate is impossible when a load of tryptophan is imposed on a pathway which is already saturated with endogenous substrate. Thus a tryptophan load greatly increases the urinary excretion of indole-3-acetic acid, a metabolite of both the decarboxylative and transaminative pathways (26). In man the daily excretion is comparable to that of 5-hydroxyindoleacetic

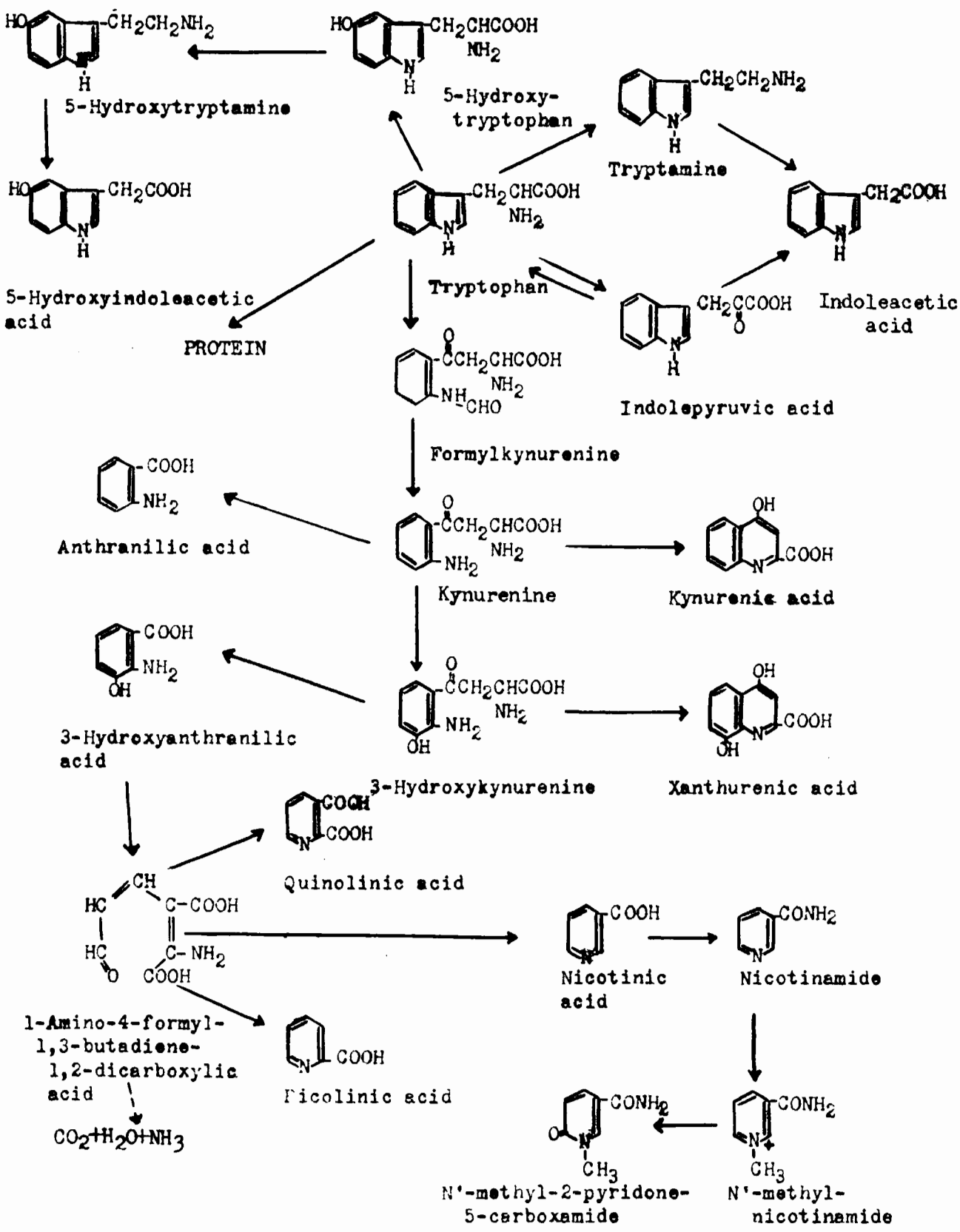

Fig. 1. Intermediary Pathways of Tryptophan Metabolism

acid (26). However this increase accounts for only 0.1% of the tryptophan load and would indicate that these are minor pathways. Other indole acids have recently been identified in human urine (27). One to 3% of dietary tryptophan has been estimated to pass through the 5-hydroxyindole pathway (28,29). Thus the preponderance of tryptophan appears to be divided between incorporation into protein and the kynurenine-forming pathway. In a study of urinary metabolites from four humans ingesting a constant diet, Price, Brown and Ellis found that kynurenic acid, xanthurenic acid, kynurenine, N^{α}-acetylkynurenine, o-aminohippuric acid, anthranilic acid glucuronide and an unknown diazotizable amine account for 2.5% of the dietary tryptophan (30). When tryptophan labelled with N^{15} in the indole ring is administered to rats only 11 to 18% of the ingested N^{15} appears as urinary urea and ammonia (31). The urinary ammonia was found to have an isotope concentration four times as high as the urinary urea indicating that the kidney is probably the principle site of the ring-splitting reaction. N^{15}-containing amino acids isolated from the internal organs of a rat demonstrated that the ring nitrogen of tryptophan can enter the general nitrogen pool. The tremendous capacity of the metabolic pathway via 3-hydroxyanthranilic acid and acroleyl fumaric acid for degrading tryptophan is exemplified in the following experiments. Gholson et al. (32) administered DL-tryptophan-7α-C^{14} to rats. After 12 hours 36% of the C^{14} had been expired as $C^{14}O_2$, 7-15% had been excreted in the urine and 40-50% could be accounted for in the tissues. Of the latter, the majority was found in the carcass proteins, the rest in liver proteins, humin, and carcass and liver lipides. However after the administration of

carboxyl-labelled 3-hydroxyanthranilic acid to rats 60 to 66% of the C^{14} is expired as $C^{14}O_2$ within three hours and 20 to 30% of the C^{14} appears in the urine within eight to twelve hours (33). 3-Hydroxyanthranilic acid, which is only a step away from the alternate pathways to nicotinic acid and complete oxidation, gives rise to a greater amount of CO_2 in a short time than does tryptophan.

A D-amino acid oxidase preparation from rat liver and kidney has been found which oxidatively deaminates D-tryptophan to indolepyruvic acid (34). The activity of this enzyme would explain the effectiveness of D-tryptophan in replacing the L-isomer in the diet of growing rats (12,13,14) if indolepyruvic acid were to be reaminated to L-tryptophan. That this occurs is demonstrated by the fact that indolepyruvic acid is also an effective replacement for tryptophan in the diet of rats (18). Lin, Civen and Knox have reported a new enzyme, tryptophan-α-ketoglutarate transaminase, by which tryptophan yields 3-indolepyruvic acid (35). This enzyme requires pyridoxal phosphate as coenzyme and the apoenzyme is increased in activity by the administration of tryptophan to the animal (35,36). Weissbach *et al.* (26) have recently found that the formation of indole-3-acetic acid from tryptophan occurs mainly through the transaminative pathway to indolepyruvic acid followed by decarboxylation. Oxidative deamination of tryptophan to tryptamine does occur but only to a limited extent.

ii) The Kynurenine Pathway

In 1931, Kotake and Iwao (37) isolated kynurenine from the urine of rabbits fed tryptophan. The pyrrol ring-splitting reaction was found in 1936

by Kotake and Masayama (38) to be mediated by rat liver slices and extracts. These investigators found that tryptophan gives rise to "prokynurenine" which on hydrolysis yields kynurenine. These products are undoubtedly the formylkynurenine and kynurenine of the tryptophan peroxidase-oxidase system studied by Knox and Mehler in 1950 (39,40). The name, tryptophan pyrrolase, has now been adopted by Knox and his co-workers (41) in view of recent findings on the mechanism of this reaction.

One of the metabolites of tryptophan degradation, kynurenic acid, was isolated from the urine of dogs as early as 1853 by Liebig (42). It was related to tryptophan by Ellinger in 1904 (43). Subsequently indole-3-pyruvic acid (44,45,47) and indole-3-lactic acid (45) were found to give rise to urinary kynurenic acid. In 1931, Kotake and his group (37) showed that kynurenine injected into rabbits gives rise to urinary kynurenic acid. Matsuoka et al. found no evidence for the conversion of D-tryptophan to kynurenic acid (46); this was also noted by Borchers, Berg and Whitman (47). The latter investigators surmised that kynurenine is an intermediate in the metabolism of the large proportion of tryptophan which is not eliminated as kynurenic acid in 1942 (47). They based this hypothesis on their observations of the urinary output of kynurenic acid, and kynurenine after feeding tryptophan or kynurenine.

Another kynurenine metabolite is anthranilic acid (See Fig. 1). The formation of anthranilic acid through the mediation of the enzyme, kynureninase, was first observed in 1941 by Kotake and Nakayama (48). They found that a cell-free preparation from cat liver splits L-kynurenine to

anthranilic acid. After the administration of tryptophan to a cat a considerable amount of anthranilic acid but no kynurenic acid was found in the urine. Brown and Price (49) more recently were unable to demonstrate kynurenic acid in the urine of the cat.

Wiss (50) found that the formation of anthranilic acid and kynurenic acid from kynurenine occur independently. Kynurenic acid formation results from transamination with α-ketoglutarate in the presence of a liver mitochondrial enzyme which requires pyridoxal phosphate (50,51). Kynureninase, which also requires pyridoxal phosphate for activity, is found in the soluble liver protein (50,51). Heidelberger et al. confirmed the earlier findings of kynurenine and kynurenic acid production from tryptophan in isotope studies (52). They found that the β-carbon of tryptophan becomes the β-carbon of kynurenine and the 3'-carbon of the quinoline ring of kynurenic acid.

The hydroxylation of kynurenine to 3-hydroxykynurenine by rat liver mitochondria (53,54) has been shown to be mediated through a specific oxygenase (55). Part of the side chain alanine, of 3-hydroxykynurenine is then removed through the action of kynureninase to form 3-hydroxyanthranilic acid (56). 3-Hydroxykynurenine may also be transaminated, by the same enzyme which is responsible for the transamination of kynurenine, to xanthurenic acid (50,51). Xanthurenic acid was first isolated from the urine of rats fed fibrin-rich diets by Musajo (57). In 1943 Lepkovsky et al. isolated xanthurenic acid from the urine of pyridoxine deficient rats (58). Furthermore the latter group demonstrated its origin from tryptophan since dietary tryptophan deficiency eliminates urinary xanthurenic acid excretion.

4,8-Dihydroxyquinoline has also been shown to be a metabolite of 3-hydroxykynurenine through the mediation of mouse liver homogenates, possibly through the intermediate formation of 3-hydroxykynurenamine, the decarboxylation product of 3-hydroxy-L-kynurenine (59).

Riboflavin is thought to be involved in the degradation of tryptophan owing to the altered urinary metabolite pattern which is observed after the administration of tryptophan to riboflavin-deficient animals. There is a greater conversion of tryptophan to anthranilic acid (60) and to xanthurenic acid (61,62), but less conversion to quinolinic acid, a metabolite below the level of 3-hydroxyanthranilic acid (62). While in riboflavin deficiency more of the administered tryptophan is diverted to the formation of xanthurenic acid, less of administered 3-hydroxykynurenine, the immediate precursor, is diverted to this product than in the normal animal (62). This evidence does not favour a role for riboflavin in the hydroxylation of kynurenine because more of the dihydroxylated quinoline derivative, xanthurenic acid is found in the deficient animal than the normal one. Helmann and Wiss (63,64,65) isolated 3-hydroxyanthranilic acid phosphate after the incubation of rat or hog liver homogenates with tryptophan, kynurenine or anthranilic but not after incubation with 3-hydroxykynurenine or 3-hydroxyanthranilic acid. The significance of this phosphoric acid ester is not known. Charconnet-Harding *et al.* have suggested that riboflavin may be involved in the oxidative phosphorylation of this substance (61). If 3-hydroxykynurenine phosphate were the physiological substrate for kynureninase then more kynurenine would be available in riboflavin deficiency for shunting via the

xanthurenic acid pathway (61). This explanation does not explain however the decreased conversion of administered 3-hydroxykynurenine to xanthurenic acid in riboflavin deficiency (62).

Both the kynureninase and kynurenine transaminative enzymes require pyridoxal phosphate for their activity. The latter enzyme is less easily resolved with respect to its coenzyme and when tryptophan is administered to a pyridoxine deficient animal the substrates of kynureninase and their conjugated derivatives and the products of the transaminative pathway, kynurenic acid and xanthurenic acid are excreted (66,67).

Many dietary and isotope investigations have clarified the finding of Krehl et al. in 1945 (68) that rats fed a diet deficient in nicotinic acid grow if given tryptophan. The 3'-carbon of tryptophan has been shown to form the ɣ-carbon of kynurenine and to appear in the carboxyl group of anthranilic acid and nicotinic acid (69). 3-Hydroxyanthranilic acid was shown to be a more immediate precursor of nicotinic acid than tryptophan (70) and evidence for the formation of an intermediate oxidation product of 3-hydroxyanthranilic acid was found (71,72). It was only in 1956 that Wiss reported the presence of a liver enzyme which cleaves 3-hydroxyanthranilic acid oxidatively to yield 1-amino-4-formyl-1:3-butadiene-1:2-dicarboxylic acid (73,74). This intermediate can be completely oxidized in vivo or, alternately, condensation of the intermediate can occur to form quinolinic acid (75,76,72,77). The decarboxylation and condensation of the intermediate through the mediation of picolinic decarboxylase results in the formation of picolinic acid (78,79,80). Seven to twenty-four percent of

labelled quinolinic acid has been recovered as N' -methyl nicotinamide (81,82).

The conversion of nicotinic acid to nicotinamide was first demonstrated in 1948 by Ellinger (83). Later a nicotinamide methylkinase from rat liver was found to methylate nicotinamide to N'-methylinicotinamide (84), and this product is further oxidized to N'-methyl-2-pyridone-5-carboxamide by a liver flavoprotein (85,86,87).

iii) The 5-Hydroxyindole Pathway

The 5-hydroxyindole pathway is one of great pharmacological and physiological significance to the organism. Two groups working independently are responsible for elucidating this pathway. Erspamer and his colleagues as early as 1937 began their investigations of the substance, which they called "enteramine", secreted by the enterochromaffin cell system of vertebrates (88,89,90,91,92,93). In 1947, Rapport, Green and Page reported the partial purification of a vasoconstrictor substance from beef serum (94). They crystallized the substance and called it "serotonin" because of its source and activity (95,96,97). Rapport completed the characterization of the isolated creatinine sulphate complex and established the empirical formula (98). 5-HT (5-hydroxytryptamine) was first synthesized in 1951 and found to be identical with serotonin (99,100) and enteramine (101,92).

The discovery of a new and seemingly important pathway of tryptophan metabolism soon captured the imagination of many groups. Ek and Witkop synthesized 5- and 7-hydroxytryptophan and found that only the former possesses

pressor activity. Moreover they ascertained that 5-HTP (5-hydroxytryptophan) is not a substrate for the kynurenine-forming system and from these facts, concluded that the 5-hydroxyindole pathway represents a route distinct from the kynurenine-forming pathway (102). Udenfriend, Clark and Titus found that the aerobic incubation of C^{14}-tryptophan with liver slices in the presence of non-isotopic 5-HTP yields 5-HTP which contains 5% of the radioactivity (103). Dalgliesh and Dutton, however, were unable to detect any conversion of tryptophan to 5-hydroxyindoles in experiments employing rat and mouse liver homogenates and slices or rat liver perfusion (104). The mechanism by which tryptophan or a derivative of tryptophan is hydroxylated in the 5'-position still remains obscure.

Tryptophan is the dietary precursor of 5-HT: the administration of tryptophan-low diets to rats for two weeks has been found to cause significant decreases in their intestinal content of 5-hydroxytryptamine (105). Studies with patients bearing the serotonin-producing carcinoid tumour have also substantiated this. The administration of C^{14}-tryptophan to patients was found to lead to the excretion of isotopic 5-HIAA (5-hydroxyindoleacetic acid), a metabolite of 5-HT (106,29). In one patient 60% of the dietary tryptophan could be accounted for in urinary 5-hydroxyindoles, whereas only 1 to 3% is normally metabolized in this way (29,28). Isotopic 5-HT can be isolated from the blood platelets (107[a],107[b],108), a spleen, stomach and intestine of rabbits after several days of intraperitoneal administration of isotopic tryptophan (108). However, the oral administration of large quantities of tryptophan to dogs has been found to have no significant

effect on the urinary excretion of 5-HIAA (107[b]).

The possibility of tryptamine serving as an intermediate in the formation of 5-HT has not been overlooked. Attempts to demonstrate its formation from tryptophan in a large number of tissues were hitherto unsuccessful (28). Recently, however, kidney and liver homogenates have been found capable of decarboxylating tryptophan to yield tryptamine (26). Administered intravenously tryptamine was not found to give rise to an increased excretion of 5-HIAA (107[b]).

The role of 5-HTP as an intermediate in 5-HT biosynthesis has been substantiated in two ways. The first is the observation by Sandler and Snow of an atypical carcinoid tumour which secretes 5-HTP (109). Lembeck had already attributed the symptoms of a carcinoid tumour, argentaffinoma, to its secretion of 5-HT (110). The atypical tumour consisted essentially of argyrophil cells and these authors suggested that these cells may form 5-HTP. The second piece of evidence for the existence of 5-HTP as an intermediate is the widespread distribution of an enzyme decarboxylating it. This enzyme was first found in liver and kidney (103), but is also present in the gastro-intestinal tract and brain (111,112). Only the L-isomer of 5-HTP is decarboxylated to yield 5-HT (113,114). Tryptophan, 7-hydroxytryptophan (113,114), tyrosine and phenylalanine are not attacked (114). This enzyme is inhibited by carbonyl reagents (114,116) and chelating agents (115). In pyridoxine deficiency the activity of the rat kidney enzyme is reduced (116) and in chickens, pyridoxine deficiency produces a considerable reduction of the 5-HT levels of the brain, intestine and blood (117). From this evidence

it seems probable that pyridoxine serves as the coenzyme, pyridoxal phosphate, for 5-HTP decarboxylase. Consistent with the distribution of 5-HTP decarboxylase are the pharmacological effects elicited by administering 5-HTP. Whereas 5-hydroxytryptamine barely passes the blood-brain barrier, the amino acid readily passes through and elevates the brain content of 5-hydroxytryptamine as much as twenty-fold (118,119,120). Elevated levels of 5-HT can be sustained by chronic administration of the amino acid with the resultant pharmacological effects of diarrhea (121) and vasomotor disturbance (121,122). One group of investigators reported finding a carcinoid tumour containing about one thousand times the normal tissue concentration (appendix tissue) of 5-HT and thirty-five times the normal concentration of the decarboxylase (123).

5-HT was first isolated from the gastrointestinal tract (93) and serum (94). In the gastrointestinal tract it is concentrated in the intestinal mucous membrane (92,124). Twarog and Page found 5-HT in dog and rat brain (125), an organ in which its distribution has been mapped out by Amin, Crawford and Gaddum (126). It is also present in the nervous system of invertebrates (127). To a great extent 5-HTP decarboxylase parallels the distribution of 5-HT in the brain (112,128) which would seem significant if the former were instrumental in the formation of the latter. In blood 5-HT is restricted to the platelets (129,107[a]); these do not contain 5-HTP decarboxylase (107[a]).

That 5-HT metabolism gives rise to urinary 5-HIAA was first observed in 1953 by Udenfriend et al. (114). Subsequently it was found that liver and

and kidney homogenates can carry out this conversion in vitro (130). Semicarbazide blocks this reaction which is considered to be mediated through monoamine oxidase. Erspamer, in 1942, had found that the inactivation of "enteramine" was apparently due to the action of amine oxidase (131,132). 5-HT and monoamine oxidase are most highly concentrated in the hypothalamus but, whereas 5-HT is localized in the primitive portions of the brain, monoamine oxidase is widely distributed (133). On the basis of experiments on the fate of exogenously administered 5-HT Erspamer has estimated that only one third of endogenous 5-HT is metabolized and excreted as 5-HIAA in the rat (134,135) and in humans (135), and less than one half in the dog (135). 5-HIAA can also be detected in the blood after parenteral administration of high doses of 5-HT (135). Erspamer considers that the 5-HT unaccounted for as 5-HIAA may well undergo rupture of the indole ring and hence be excreted in some other form. The results of Udenfriend et al. are in agreement as they found with liver and kidney slices and homogenates only a 30% conversion to 5-HIAA (28,107^{b}). They also found that the oral or intravenous administration of 5-HT or the corresponding amino acid to dogs can be accounted for as 5-HIAA only to the extent of 20 to 30% (28,107^{b}). Recently it was demonstrated that nearly all the C^{14}-5-HT administered to Marsilid -treated mice is excreted as the o-glucuronide (136). This pathway was found to account for at least 25% of administered 5-HT in normal animals. A carcinoid urine has revealed the presence of the creatinine sulphate complex of 5-HT, 5-HIAA, 5-hydroxyindoleaceturic acid and N-acetyl-5-hydroxytryptamine (137). These metabolites as well as two others (one being the o-glucuronide)

were found in the urine of rats and rabbits fed isotopic 5-HT (137).

Udenfriend and Weissbach (108) have obtained some data on the turnover of 5-HT in rabbits. These data are based on the fall in specific activity of tissue 5-HT after the administration of isotopic tryptophan and 5-HTP. Table I summarizes these findings.

Table I

Turnover of 5-HT in the Tissue of the Rabbit*

Tissue	Half-life (hours)
Platelets	33-48
Spleen	33-48
Stomach	10
Intestine	17

*Udenfriend and Weissbach (108)

Following the administration of harmaline, a monoamine oxidase inhibitor, these investigators found that the brain 5-HT in rats is doubled in thirty minutes (108). This finding suggests that the half-life of brain 5-HT is of the order of minutes.

Erspamer has also provided some information on the metabolism of endogenous 5-HT by the rat (134,134). These data are summarized in Table II.

Table II

Quantitative Estimation of the Metabolism of 5-HT in the Rat*

Organ	µg. 5-HT/Kg.
Whole organism	125
Gastrointestinal tract	80
Blood	34
Spleen	11
As urinary 5-HIAA/24 hrs.	75
Equivalent of 5-HT metab./24 hrs.	225

*Erspamer (134,135)

The value of 75 µg. 5-HT excreted as 5-HIAA is multiplied by three to give an estimate of the total endogenous 5-HT metabolized because only one third of endogenous 5-HT is considered to be converted to 5-HIAA. From these data it can be seen that an amount of 5-HT corresponding to the total content of the rat is metabolized every twelve to fourteen hours.

In 1955 it was discovered that the administration of reserpine to animals causes the liberation of 5-HT from the intestine (138), brain and blood platelets (139,140,141) concomitant with the elimination of large quantities of 5-HIAA. Reserpine seems to impair the capacity of the cells to bind 5-HT, for its effect persists after it has disappeared from the tissues (141,142,143). The primary action of reserpine appears to be the impairment of binding sites because it does not interfere with the biosynthesis of 5-HT (144) or the conversion of 5-HTP to 5-HT (145,146).

E. TWO ENZYMES OF IMPORTANCE IN TRYPTOPHAN METABOLISM

i) Tryptophan Pyrrolase

Until recently the mechanism for the oxidation of tryptophan to formylkynurenine was thought to be a coupled peroxidase-oxidase reaction (39,147). This mechanism has lately been refuted by Knox (41). In 1958 Tanaka and Knox established that the enzyme responsible for this oxidation contains an iron-porphyrin prosthetic group which is reduced from the inactive ferric form to an active ferrous form by the action of peroxide and tryptophan (148,41). The active ferrous protoheme enzyme oxygenates tryptophan without valence change and without stoichiometric formation or utilization of peroxide (41). For this reason Tanaka and Knox have decided to adopt the designation "tryptophan pyrrolase" of Itagaki and Nakayama (149) in favour of "tryptophan peroxidase-oxidase" introduced by Knox and Mehler in 1950 (39).

During the course of the oxidation of tryptophan by TP (tryptophan pyrrolase) two atoms of atmospheric oxygen are incorporated at the 2'- and 3'-positions of the indole ring (150) and the ring is ruptured between these two positions with the production of formylkynurenine. Formylkynurenine is then hydrolysed by a distinct enzyme, formamidase (formylase), to formate and kynurenine (40). This enzyme is present in a six-hundred fold excess over TP in the liver (156). α-hydroxytryptophan (151,152,38,66) and α,β-dihydroxytryptophan (150) are not intermediates in this oxidation, as they have been shown to be inactive in this system. However, the recent finding that peroxide is not an active participant in the formation of formylkynurenine suggests that the intermediate may be a short-lived oxygen-tryptophan eddition complex.

Inhibitors of TP are cyanide, hydrogen sulfide, arsenite, fluoride,

hydroxylamine, azide (39), copper sulphate and carbon monoxide (147). Cyanide does not inhibit however when it is added after the initiation of the reaction. The enzyme system is very unstable in the absence of its substrate (39,155). The addition of autoxidizable dyes, adenylic acid and magnesium have been found to reactivate aged or dialysed preparations to a considerable extent (39). ∝-Methyltryptophan, like tryptophan, stabilizes the labile enzyme system (154). The dissociation constant (K_s) of TP using L-tryptophan is 4×10^{-4} M (39).

TP has been found only in the liver of mammals and is highly substrate-specific. Only the L-isomer of tryptophan gives rise to formylknurenine (39). 5-Hydroxytryptophan and 7-hydroxytryptophan are not degraded by this enzyme (102,153); they inhibit the degradation of L-tryptophan competitively (153). The ∝-amino group and ∝-hydrogen atom are essential for activity because acetyl-DL-tryptophan (39) and ∝-methyltryptophan (154) are not attacked by TP. ∝-Methyltryptophan also inhibits the degradation of the natural substrate. The 4-,5-,6-, and 7-methyl derivatives of tryptophan are not oxidized by bacterial TP but are much less inhibitory than the 5- and 7-hydroxy derivatives (153). Other indole compounds which are not degraded by TP are tryptamine, indole-3-acetic acid, indole-3-propionic acid, indole-3-butyric acid, skatole, oxindole, 1-ethyloxindole, dioxindole, acetonyl-3-hydroxyindole, isatin, 1-methyl-DL-tryptophan (39) and hypaphorine (154). This evidence indicates the strict specificity of TP for the integrity of both the benzene and side chain moieties of tryptophan.

TP possesses a property rarely known amongst mammalian enzymes,

that of adaptive formation. The administration of tryptophan to the animal before sacrifice and assay of the liver enzymes results in a considerable increase in the activity of TP (39). This increase in activity follows the oral, subcutaneous or intraperitoneal administration of tryptophan (156):

Rabbits		
Control (9)	1.4 ± 0.3	μM. Kyn. formed/g. liver/hr.
5-12 hrs. after 10 mM DL-trypt. (10)	11.7 ± 1.3	"
Rats		
Control (22)	1.2 ± 0.2	"
4-10 hrs. after 1-2 mM DL-trypt. (7)	8.0 ± 1.8	"

The response is linear in fashion up to a dosage of 0.5 mMole per 100 grams rat according to Lee (157) and 0.1 mMole per 100 gram rat according to Schor and Frieden (158). The rate of increase is independent of the dose but the maximal increase is achieved at the higher dosage levels at a time when increases due to lower dosages are falling off (157). The time of the peak may depend on the gradual uptake of tryptophan from the blood, since Lee has shown that the accumulation of tryptophan in the blood parallels the increase in liver TP while the liver itself does not accumulate tryptophan (157). Schor and Frieden's results may not represent the true peak increases for the reasons just mentioned. The increase in TP may amount to as much as ten times the basal level before induction (39,156). The isolated perfused liver of the rat or rabbit has also been shown to respond to the administration of tryptophan to the perfusate. A three-fold increase in the activity of liver TP has been achieved in this way (159). Two-fold increases in the activity of liver slices can be achieved by incubation of the slices in a medium

containing tryptophan (160,161).

The activity of TP has been studied in the developing liver of guinea pigs and rats. In the guinea pig TP activity first appears at about the fifty-second day of gestation and increases rapidly after birth (162). The activity of TP however cannot be increased by the administration of tryptophan until the fetus is at term. Twenty-four hours after birth the activity of TP can be increased to the same extent as in adult guinea pigs. In the rat TP is not demonstrable until the twelfth day after birth. With administration of tryptophan, it is demonstrable on the third day (163). Two of three week old rats do not respond to the same extent as adults to the administration of tryptophan.

While TP is highly substrate-specific, it does not appear to require the same specificity in the form of its inducer. α-Methyltryptophan is an even more potent inducer of TP than tryptophan (154):

Intact rat		
saline control	0.24	μMole Kyn./hr./60 mg. dry liver
0.5 mM. DL-trypt.	1.28	"
0.5 mM. DL-α-methyltrypt.	2.83	"

This fact can probably be attributed to the concentration of α-methyltryptophan in the cell because it is not degraded while the natural substrate serving as an inducer is also degraded.

The activity of TP is also influenced by hormonal factors. Adrenalectomy (Adrex-Table III, A) is followed by a considerable decrease in the constitutive level of TP activity (164,165,158,185). Thomson and Mikuta (Table III, B) did not find this decrease in their adrenalectomized rats

but this may be due to higher levels of dietary tryptophan (168). Adrenalectomized rats respond normally to the administration of tryptophan by an increase in their liver TP activity.

Table III

The Response of Intact and Adrenalectomized (Adrex) Rats to Various Treatments

A*	uMole Kyn./hr./g. dry liver				
	-	Cortisone	Histidine	Cortisone + Histidine	ACTH
Intact	5.7±0.34	14.3±1.21	19.6±1.33	18.3	8.3±0.85
Adrex (4 days)	4.6±0.36	12.7±1.31	5.9±0.64	18.7±1.10	-

B#	Days	-	Total Body x-irradiation	Hydrocortisone	Tryptophan
Intact	-	7.7±1.5	13.9±2.4	15.5±3.4	121±18
Adrex	7-9	7.4±1.9	7.0±1.3	17.1±2.4	-
Ad-demull.[a]	21-28	8.1±1.5	11.4±1.1	12.1±2.3	74±25
Thyrex[b]	35	6.8±1.4	14.1±1.2	13.7±0.5	81±14
Hypophysex[c]	7	6.8±0.1	9.6±0.2	-	-
"	14	5.4±1.2	6.6±1.3	23.0±5.3	25±12
" - ACTH	14	6.8±0.6	9.0±1.0	24.0±2.9	35± 3

* Knox and Auerbach (165)
\# Thomson and Mikuta (168)
a Adrenal demedullated
b Thyroidectomized
c Hypophysectomized

The administration of histidine, tyrosine, phenylalanine and kynurenine increases the activity of TP in intact rats (156) but does not affect the activity in adrenalectomized rats (164). These substances are therefore considered to act through stimulation of the adrenal cortex (164) (histidine - Table III A). Table III A denotes the lack of summation in inducing effect of approximately equally effective doses of cortisone and histidine in the intact rat. Even adrenaline which is an effective inducer in the intact rat is ineffective in the adrenalectomized one (164). X-Irradiation (Table III B) doubles the activity of TP in intact rats in tour hours but this effect too is mediated through the adrenals because it is eradicated in hypophysectomized or adrenalectomized rats. The administration of corticotrophin (ACTH) also increases the activity of TP but is not as effective as cortisone (Table III A). Hydrocortisone is an even more effective inducer than cortisone in adrenalectomized rats (166). Geschwind and Li (167) have shown that adrenalectomy results in a decrease of 25% in the induced enzyme level of TP while hypophysectomy results in a 35% decrease (Thomson and Mikuta's results (Table III B) show a more striking effect). ACTH effects only a small increase after hypophysectomy in the induced level of TP (167) (Table III B). Geschwind and Li found that growth hormone, thyroxine or ACTH effect a considerable increase in the lowered constitutive levels of TP found in thier hypophysectomized rats (167). Both groups of investigators found similar decreases in the response to tryptophan in thyroidectomized rats (Table III B) but Geschwind and Li also found an increase of 174% in the constitutional level of TP.

These variable results are no doubt due to the difficult surgery involved in totally ablating these glands in rats. They do signify nevertheless the hormonal control over the constitutional and adapted levels of this enzyme. Recently it has been found that TP increases in activity in rats during a week of inanition (169).

Insulin has also been found to be an effective inducer of TP (158): it is effective in the adrenalectomized rat. The injection of alloxan to rats causes a biphasic increase in TP activity. The first peak of activity, which is known to be mediated through the adrenals, occurs six hours after the injection of alloxan. The second peak occurs at seventy-two hours and can be reduced to normal levels by the administration of insulin in a dosage too low to be an effective inducer but almost sufficient to control the diabetes. Cortisone plus insulin or tryptophan plus insulin administered simultaneously to rats yields TP activities which are higher than the activity due to the administration of each substance singly. This effect, as interpreted by Schor and Frieden, may be due to an increased availability of tryptophan from two sources. One source is the tryptophan injected or the tryptophan released with other amino acids in protein catabolism caused by cortisone. The second source is possibly the increased accessibility of tryptophan due to changes in cell permeability through the action of insulin.

It is possible that the apparent adaptation emanates from protection by the substrate of a rapidly metabolized enzyme (high turnover). However many arguments favour the concept that TP is a truly adaptive enzyme, that is, that the increased activity of the liver enzyme following induction is

due to the formation of new enzyme protein. The enzyme activity is measured in a cell-free preparation so that permeability changes cannot account for the increased activity after induction (164). The possibility that a simple inhibitor exists in uninduced liver or that an activator exists in induced liver is eliminated by the summation of their activities when mixed homogenates are assayed (156,168). Furthermore histological examination has revealed that there is no rapid mitotic activity concurrent with the induction phase (157) so that an increase in the mass of cells cannot explain the increase in activity.

The inhibitory action of the antimetabolite, ethionine, is interpreted by many investigators as an interference with the incorporation of amino acid precursors into enzymatic protein. Methionine relieves this inhibition presumably by competition with ethionine. Recently Farber and Corban (172) have demonstrated that ethionine administration produces a 50% reduction in the constitutive level of TP in female rats but is without effect on the constitutive level in male rats. The simultaneous administration of methionine and ethionine causes a 100% increase in the constitutive level of the female liver TP. The administration of ethionine has been found to inhibit the adaptive increase in TP following induction with tryptophan in intact and adrenalectomized rats (170). Methionine relieves this inhibition, which is interpreted as due to the interference of ethionine in the utilization of methionine for enzyme synthesis. In liver perfusion studies, where the addition of tryptophan to the perfusate induces an increased TP activity, ethionine inhibition and methionine relief of the inhibition can also be

observed (159). Ethionine interferes with the TP adaptive response to cortisone but this too is relieved by the administration of methionine (171).

The administration of isotopic cystine to rats before induction with tryptophan has shown that the increase in TP activity is accompanied by a change in the rate of protein turnover of all structural elements of the cell (173). In rats and rabbits administered isotopic valine fourteen hours before induction with tryptophan, the liver TP fraction contains a high specific activity (174). The specific activity of TP after induction is about four times the specific activity of the serum proteins. After γ-irradiation, from the fourth to sixteenth day when the constitutive level of TP is increased, the response to tryptophan is less than half that of control animals and the incorporation of isotopic valine is about a third of the incorporation by controls. In female rats treated with ethionine, concurrent with the decrease in their constitutive level of TP, there is also a decreased incorporation of isotopic leucine, valine and glycine into the total liver protein (172).

Threonine dehydrase is another 'adaptive' enzyme whose induction is inhibited by ethionine (175). In perfusion experiments, a complete amino acid mixture is required for the induction to be observed. This requirement suggests the necessity of an amino acid pool for de novo synthesis of the enzyme protein. Interesting in this respect was the recent finding of Chytil that the simultaneous administration of tryptophan and threonine results in a greater activity of TP than after induction with tryptophan alone (176). It is tempting to conjecture that the inducers mobilize independently a greater amino acid pool than they do individually, and this pool is utilized

more efficiently for TP synthesis. Chytil does not report the activity of threonine dehydrase after the administration of both amino acids.

In 1958 Feigelson et al. reported their studies of the turnover of RNA following tryptophan administration to rats (177). They found that P^{32} incorporation into RNA occurs at control rates during the period when TP activity is increasing but that RNA turnover is increased to three times the control values at the time when TP activity is falling to control levels. The majority of the increased RNA turnover occurs in the microsomal fraction where it is known that amino acid incorporation into protein takes place. These investigators suggested that an RNA template for TP synthesis is utilized during the period of increased TP activity (protein synthesis) and is subsequently replenished. Lee has found that TP adaptation is accompanied by a reduced incorporation of P^{32} into the nuclear protein nucleic acids while the incorporation into cytoplasmic nucleic acids remains at control levels (178). The administration of the LD_{50} dose of various nucleic acid antagonists twenty-four hours before the administration of tryptophan was found to result in TP activity 160 to 250% greater than that of untreated animals. Lee, too, suggested a reciprocal relationship between nucleic acid metabolism and the adaptation of TP in the liver.

In 1948, Junquiera and Schweigert found that thiamine deficiency decreases the conversion of tryptophan to nicotinic acid derivatives in the rat (179). On the basis of studies on the urinary excretion pattern of rats on thiamine, pyridoxine, and doubly deficient diets, Dalgliesh drew the conclusion

that thiamine is involved at the TP stage of tryptophan degradation (180,181). The administration of a tryptophan load to pyridoxine-deficient rats results in the excretion of the products of TP and kynurenine transaminase but not those of kynureninase whose coenzyme is easily resolved by the deficiency. When a thiamine deficiency is imposed simultaneously, the metabolites characteristic of the pyridoxine deficiency appear first in the urine, then, as the thiamine deficiency progresses, they too cease except for a small excretion of xanthurenic acid (180). The administration of thiamine to the doubly deficient rats results in the re-appearance of the pyridoxine-deficiency excretory pattern. When kynurenine is given to the doubly deficient animals there is a considerable excretion of kynurenine, 3-hydroxykynurenine and xanthurenic acid. None of these metabolites is excreted in thiamine-deficient rats administered tryptophan which suggests that a block is incurred at the TP level. Even control rats administered tryptophan excrete traces of kynurenine, anthranilic acid and xanthurenic acid. Porter, Clark and Silber (182) found that the total amount of administered tryptophan excreted as kynurenine, hydroxykynurenine, kynurenic and xanthurenic acids is not significantly different in mildly thiamine-deficient rats from that in control animals. However, of these products more xanthurenic acid is excreted at the expense of kynurenic acid in thiamine-deficient rats as compared to the control rats.

The effect of thiamine deficiency on TP has been studied enzymatically. Ginoulhiac (183) examined the TP activity of rats maintained on thiamine-deficient diets and could find no significant change in the TP activity even when the animals suffered from polyneuritis (Table IV - ref. a). This was

confirmed by Mangoni et al. (184) who also found that the adaptation to tryptophan was unaffected by thiamine deficiency. Hormonal adaptation of TP is unaffected by dietary thiamine (Table IV - ref. b).

Table IV

Tryptophan Pyrrolase Activity in Thiamine-deficient and Thiamine-sufficient Rats

Treatment	mg. Kyn./100 mg. N_2/hr. B_1-deficient	B_1-sufficient
Intact Rats		
-	2.38[a]	1.05[a]
-	2.27 ± 0.4[b]	2.03 ± 0.25[b]
-		2.12 ± 0.33[c]
Prednisone	7.19 ± 1.27[b]	5.40 ± 1.48[c]
Adrenalectomized Rats		
-		1.14 ± 0.43[b]
-	0.74 ± 0.26[b]	1.02 ± 0.15[c]
Prednisone	5.58 ± 2.86[b]	9.69 ± 2.64[c]

a Ginoulhiac - Ref. 183
b Ginoulhiac and Tenconi - Ref. 186
c Tenconi - Ref. 185

As will become evident in the experimental part of this thesis experiments on the effect of thiamine deficiency on the constitutive and adapted levels of TP form a part of the present study. These experiments were in progress at the time that Ginoulhiac's results (183) were published. The results of Mangoni et al. (184) who studied the effect of thiamine deficiency on TP induction by tryptophan in intact rats (not adrenalectomized) were published in 1957 and only recently came to the attention of our laboratory through Chemical Abstracts.

ii) Kynureninase

Kynureninase activity is essential for the degradation of tryptophan by what appears to be the major pathway of its metabolism. The enzyme catalyses the cleavage of alanine from the side chain of kynurenine and 3-hydroxykynurenine

with the resultant formation of anthranilic and 3-hydroxyanthranilic acids, respectively. The first observation of this transformation in mammals was made by Kotake and Nakayama in 1941 (48). These investigators found that although cats rapidly degrade kynurenine they do not excrete kynurenic acid even after the administration of tryptophan. They were able to prepare cell-free preparations from cat liver and kidney which split kynurenine to anthranilic acid.

The coenzyme requirement of kynureninase was demonstrated by Braunshtein et al. in 1949 (56). The decreased activity of this enzyme in pyridoxine-deficient rats is restored by the addition of pyridoxal phosphate to the liver or kidney enzyme system in vitro. Furthermore, kynureninase is more easily resolved with respect to its coenzyme than the kynurenine transaminative enzyme. In pyridoxine deficiency kynurenine, 3-hydroxykynurenine and their transamination products, kynurenic and xanthurenic acids, accumulate in the urine after the administration of tryptophan (66,67). Mason and Berg found that kynurenic acid formation is impaired in pyridoxine-deficient rats and that even in control animals both enzymes, kynureninase and transaminase, are not completely saturated with the coenzyme (187). Wiss found that a partially purified preparation of kynureninase loses activity on dialysis which can be restored by the addition of pyridoxal phosphate (51). The kynureninase contained in the supernatant of a rat liver homogenate is resolved to the extent of about 25% (189). One hundred percent of the activity can be achieved by pre-incubation of the enzyme preparation with pyridoxal phosphate.

The hypothesis that kynurenic acid is formed as a by-product of

anthranilic acid formation through the intermediary formation of an $\alpha\gamma$-diketo acid (66,188) was refuted by the findings of Knox and Wiss. Acylpyruvase was not found to participate in the kynureninase reaction or to diminish kynurenic acid formation in Knox's experiments (189). Wiss (50) at the same time showed that anthranilic acid formation in hog liver does not occur through deamination to o-aminobenzoylpyruvic acid but through a cleavage reaction. An active kynureninase from pork liver which was electrophoretically homogeneous and appeared pure by its behaviour in the ultracentrifuge, was prepared by Wiss and Weber in 1956 (190). This preparation cleaved the carbon linkage between the β- and γ-carbons of kynurenine hydrolytically. No activation was achieved by the addition of calcium, manganese, cobalt or zinc. Magnesium has been found to activate the bacterial enzyme (194). Both the mammalian liver enzymes (51) and the bacterial enzyme (194) cleave kynurenine and 3-hydroxykynurenine. Mammalian kidney is not as active as liver (185); Knox found that rat kidney is only about 10% as active as rat liver (189).

Mammalian liver kynureninase does not exhibit a high substrate specificity. Wiss and Fuchs found that 3-hydroxykynurenine is hydrolysed at twice the rate that kynurenine is hydrolysed (192). However α-amino-β-benzoylpropionic acid and α-amino-δ-hydroxyphenylbutyric acid are metabolized at the same rate as kynurenine. Substances such as α-amino-β-acetylpropionic acid and α-amino-γ-hydroxyvaleric acid, which do not contain the benzene ring are also attacked. The bacterial kynureninase is more specific (153). N^{α}-acetylkynurenine, a conjugated product observed in the urine of pyridoxine-deficient rats (66), is not a substrate for kynureninase until it

is deacetylated (189). D-Kynurenine is not attacked (48,189).

Rat liver kynureninase was found by Knox to be half-saturated at 4×10^{-4} M L-kynurenine (189). The values for kynureninase from Pseudomonas fluorescens (193) and Neurospora (194) are 3.9×10^{-5} and 6×10^{-6} M respectively. The dissociation constant of Neurospora kynureninase has been found to be 3×10^{-6} M using hydroxykynurenine as substrate (194). The Neurospora kynureninase is inhibited by substrate concentrations higher than 4×10^{-4} M.

Bacterial kynureninase is inhibited by cyanide, hydroxylamine (193, 194), fluoride, sulphite (194) and semicarbazide (193). This inhibition probably occurs through combination with the carbonyl group of pyridoxal phosphate. The mechanism of the interaction of kynureninase from Neurospora with its substrates and coenzyme has been studied by Jakoby and Bonner (195). Dihydroxyphenylalanine, histidine and cysteine are inhibitory because they inactivate pyridoxal phosphate by forming cyclic addition products with it. D-Kynurenine and N^{α}-acetylkynurenine are not substrates, the former inhibits the enzyme, the latter not. N'-Formylkynurenine, which is a substrate, is inhibitory. From this evidence it would seem that a Schiff base is formed through the α-amino group. (Substituted in N^{α}-acetylkynurenine and free in D-kynurenine). Excess substrate and excess pyridoxal phosphate are both inhibitory to the kynureninase reaction. This can be explained by the removal of either the substrate or the coenzyme by the other component and by the inhibitory action of the kynurenine-pyridoxal phosphate Schiff base analogue itself. The formation of the latter compound implies that this analogue is not the one which normally is attached to the apoenzyme during the reaction.

A mechanism for kynureninase action in which the Schiff base formed between kynurenine and the pyridoxal phosphate enzyme is converted to the Schiff base of α-aminoacrylic acid has been proposed by Longenecker and Snell (196).

II METHODS

A. ANIMALS

All the animals used in the experiments to be reported were male rats of the Sprague-Dawley strain. They were well acclimatized to the animal room before experimental procedures were imposed upon them. The rats were maintained on Purina chow pellets. In acute experiments animals weighing between 78 and 250 g. were used; where several animals were employed in a single experiment their weights were narrowly restricted. In the case of dietary experiments animals were selected in a narrow weight range. The weight of animals in various groups extended from 82 to 264 grams.

B. PREPARATION FOR DIETARY EXPERIMENTS

i) Randomization

Rats of a restricted weight range were randomly assigned to various diets. Randomization was carried out according to the procedure of Fisher and Yates (197) and from their tables of random numbers.

ii) Diets

Various synthetic diets were employed in different experiments. They are listed numerically below Table V for convenience and will be

referred to throughout the experimental section of this thesis by these numbers. All except one diet was composed essentially as shown in Table V. Alterations in this diet are described in the numerical list. In all cases where the percentage of amino acids or protein was altered it was replaced by an equivalent weight of sucrose.

Table V

Composition of Experimental Diets

Acid-hydrolyzed casein*	19.1%
DL-Phenylalanine	0.45
L-Tyrosine	0.45
Tryptophan (see text)	0.9
Vegetable shortening (Crisco)	13.0
Cod liver oil, U.S.P.	5.0
Salt mixture, U.S.P. XIV	4.0
Cellulose powder	2.0
Sucrose, finely ground	51.1
Vitamin mixture#	4.0
	100.0%

*This material contains no tryptophan, less than 0.1% phenylalanine, and probably less than 0.1% tyrosine.
#The vitamin mixture consisted of the following items: thiamine, 75 mg. (see text); riboflavin, 75 mg.; nicotinic acid, 500 mg.; pyridoxine, 75 mg.; calcium pantothenate, 200 mg.; biotin, 3 mg.; folic acid, 15 mg.; inositol, 5 g.; p-aminobenzoic acid, 1 g.; choline, 2 g.; vitamin B_{12} triturate in mannitol, 2.5 mg.; menadione, 10 mg. These were thoroughly mixed with 91 g. sucrose.

Diet 1. Phenylalanine and tyrosine were omitted from this diet.

Diet 2. L-tyrosine was supplemented to the extent of 1.23% in this diet; no phenylalanine was omitted.

Diet 3. Acid-hydrolysed casein was added to the extent of 19.8 g., D- or L-trypt ophan to the extent of 0.2%.

Diet 4. Acid-hydrolysed casein was added to the extent of 18.7 g., D or L-tryptophan to the extent of 0.4%.

Diet 5. Acid-hydrolysed casein was replaced by 16.5 g. of zein; DL- tryptophan was added to the extent of 1% and L-lysine to the extent of 2.5%. Phenylalanine and tyrosine were omitted from this diet.

Diet 6. Acid-hydrolysed casein was replaced by 16.5 g. of zein; DL-tryptophan was added to the extent of 0.4% and L-lysine to the extent of 2.5%. Tyrosine and phenylalanine was omitted from this diet.

Diet 7. Acid-hydrolysed casein was replaced by hot alcohol extracted (vitamin free) casein to the extent of 20% of the diet.

Diet 8. This thiamine deficient diet was obtained prepared in toto commercially.

C. 5-HYDROXYINDOLE ASSAY METHODS

i) 5-Hydroxytryptamine (5-HT)

The estimation of 5-HT according to the methods of Udenfriend, Weissbach and Clark (198) and Weissbach, Waalkes and Udenfriend (199) was tried and found to be unsatisfactory. Recoveries of added 5-HT and duplicate determinations are extremely variable within a single assay. The method adopted in the experiments to be reported here is a combination of the acetone extraction procedure of Amin, Crawford and Gaddum (126), and the solvent extraction procedure and colorimetric assay of Udenfriend et al. (198). This procedure, although tedious, gives satisfactory results; recoveries of added 5-HT range from 75 to 107% and duplicate determinations are satisfactory.

The procedure used is as follows: after sacrifice of the rat the intestine is removed, slit longitudinally, rinsed with tap water, weighed and minced with scissors. The mince is transferred to the flask of a Virtis Blendor and extracted with 20 volumes of 100% acetone at speed 30 for three minutes. The blend is allowed to stand for one hour and is then filtered. The residue left on the filter paper is re-extracted with 20 volumes of 95% acetone in the Blendor for three minutes at speed 40. This extract is then allowed to stand for one half Hour and filtered. The filter paper is rinsed twice with 5 ml. of 95% acetone and the combined filtrates are taken to dryness in a water bath at 37°C. Recovery of 5-HT and duplicate determinations are estimated by combining the acetone extracts of several intestines, dividing these up for duplicate determinations and adding 5-HT to several aliquots before taking the extracts to dryness. 5-HT standard solutions and a blank without 5-HT are also carried through the entire acetone extraction procedure.

The acetone extracts are taken up in 10 ml. of distilled water and transferred to 50 ml. glass-stoppered centrifuge tubes. The beaker which contained the extract is rinsed with 5 ml. of borate buffer, pH 10, and the washings are added to the aqueous extract. The aqueous extract is adjusted to pH 10 with solid Na_2CO_3, saturated with solid NaCL and is shaken with 20 ml. n-butanol for 5 minutes. The tube contents are decanted into a fresh tube, centrifuged and the aqueous phase discarded. The butanol phase is extracted twice more with 20 ml. borate buffer and the aqueous phase discarded. A 13 ml. aliquot of the butanol phase is then transferred to another 50 ml. glass-stoppered centrifuge tube containing 26 ml. of heptane and 3 ml. of

0.2 N HCl. The tube is shaken, centrifuged and the supernatant solvent layer is removed by aspiration. A 2 ml. aliquot of the acid layer is transferred to a fresh tube and 1 ml. each of 0.1% 1-nitroso-2-naphthol in ethanol and 0.1% $NaNO_2$ in 2 N H_2SO_4 are added. The tube is shaken and heated in a water bath at 55°C. for 5 minutes. The excess nitrosonapthol reagent is removed by two extractions with 10 ml. of water-saturated ethyl acetate. The coloured derivative of 5-HT is determined at 540 mμ in the spectrophotometer. As little as 5 μg. 5-HT can be determined by this procedure.

ii) 5-Hydroxyindoleacetic acid (5-HIAA)

The procedure employed for the estimation of urinary 5-HIAA was essentially the method of Udenfriend, Titus and Weissbach (200) as modified by MacFarlane, Dalgliesh, et al. (201). Urine was collected from three or four rats kept in a metabolism cage for varying periods of time. As rats, in particular those suffering from a dietary deficiency, do not excrete large volumes of urine, it was found beneficial either to inject saline subcutaneously or administer orally a dilute sucrose solution at the commencement of urine collection. As the rats seemed to favour the latter treatment and it was found effective, it was generally adopted. The urine was collected in a glass cylinder containing 0.5 ml. of 2 N HCl and a layer of toluene to prevent oxidation of the labile 5-HIAA.

The urine volumes were found to be extremely variable and so in each assay they were made up with water to the highest volume obtained and the 5-HIAA content of the total volume determined. A standard 5-HIAA calibration curve and recoveries of added 5-HIAA from human urine were

estimated in each assay. Recoveries ranged from 50 to 90% in all experiments but within a single assay three recovery estimations did not vary more than 10%.

The procedure is as follows: the urine samples, pure 5-HIAA solutions and water blank are made up to a volume of 10 to 22 ml. with water. This volume is shaken with 5 or 6 ml. of 0.5% 2,4-dinitrophenylhydrazine in 2 N HCl and centrifuged to remove the hydrazones. A 14 to 25 ml. aliquot of the supernatant is transferred to a 50 ml. glass-stoppered centrifuge tube containing 25 ml. of water-saturated ether. The aqueous phase is saturated with solid NaCl and the tube is shaken for five minutes and centrifuged. To 20 ml. of the ether phase transferred to a fresh 50 ml. glass-stoppered centrifuge tube is added 1.5 ml. of Na-PO_4 buffer, pH 7.0. The tube is shaken for 5 minutes and centrifuged. The ether phase is removed by aspiration and discarded. To 1 ml. of the aqueous phase is added 0.5 ml. of 0.1% 1-nitroso-2-naphthol; after mixing, 0.5 ml. of 0.1% $NaNO_2$ in 2 N H_2SO_4 is added and mixed. The tube is then heated at 37°C. for 5 minutes on a water bath. The excess nitrosonaphthol reagent is removed by two extractions with 5 ml. of water-saturated ethyl acetate. The samples are determined by their optical density at 540 mμ in the spectrophotometer. The 5-HIAA in the urine samples is estimated from the calibration curve and this value is corrected for losses of 5-HIAA during the procedure. As in the 5-HT assay with the same colour reagent as little as 5 μg. of 5-HIAA can be estimated.

iii) Reserpine Treatment

Resperine was kindly donated by the Ciba Company Ltd. as Serpasil.

The reserpine from ampoules containing 5 mg./2 ml. was diluted just before use with placebo diluent also provided by Ciba Co. Ltd. The placebo contained citric acid, benzoyl alcohol, polyethylene glycol and fluorescein dye. All injections were made intravenously into the tail vein; equal volumes of placebo and reserpine were administered.

D. TRYPTOPHAN PYRROLASE ASSAY

The assay of TP was carried out essentially by the procedure of Knox and his associates (39,165,202). A rat liver suspension aerobically oxygenates tryptophan with the resultant formation of formylkynurenine (41, 150). Formylkynurenine is hydrolysed by excess formamidase (formylase) contained in the rat liver suspension to kynurenine and formate (40,202). Kynurenine can then be determined either directly by its absorption properties (39,202) or after diazotization to a coloured product (203).

Rats were sacrificed by decapitation and the livers removed immediately. The livers were rinsed in cold tap water, blotted dry and weighed. After mincing with scissors the tissue was suspended in 1 to 12 volumes (dilution depended on the expected activity of the liver) of ice-cold 0.14 M KCl containing 0.0025 N NaOH using a chilled Teflon pestle and glass homogenizing tube. The tissue suspension was centrifuged for 25 minutes at 20,000 x g. at 2°C. An aliquot of the supernatant fraction was then incubated with tryptophan for 1 hour to determine the potential activity of the TP contained in the liver. Another aliquot was incubated without the addition of tryptophan to determine the endogenous activity of the enzyme.

Incubation of the liver preparation was carried out aerobically at 37°C. in a Dubnoff shaker. The reaction mixtures were contained in 20 ml. glass beakers. The media were as follows: 1. a final volume of 2 ml. contained 1 ml. of the supernatant fraction; 0.02 M K_2HPO_4-KH_2PO_4, pH 7.4; 2 mM L-tryptophan or water;

2. a final volume of 4 ml. contained 0.25 to 1.0 ml. of the supernatant fraction; 0.066 M K_2HPO_4-KH_2PO_4, pH 7.38; 3 mM L-tryptophan or water.

After incubation for 1 hour reaction mixture. 1. was deproteinized by the addition of 2 ml. 10% trichloroacetic acid or 2 ml. of 5% zinc acetate and 2 ml. of 0.18 N NaOH. The former precipitant was used preliminary to the diazotization procedure, the latter preliminary to the direct determination of kynurenine spectroscopically. Reaction mixture 2. was deproteinized with 2 ml. of 15% trichloroacetic acid. The reaction mixtures were then transferred to test tubes and centrifuged.

The deproteinized supernatants were assayed for kynurenine. Kynurenine was determined directly by its absorption at 360 mμ in the Beckman spectrophotometer (39). The method employed in the majority of experiments was diazotization according to the method of Bratton and Marshall (203) which gave excellent recoveries. The procedure was as follows: 1 or 2 ml. of the 5% deproteinized supernatant was diluted to 10 ml. with distilled water; 1 ml. of 0.1% sodium nitrite (freshly prepared) was added to the chilled tubes and allowed to react for 3 minutes; 1 ml. of 0.5% ammonium sulphamate was added

and allowed to react for 2 minutes; 1 ml. of 0.1% N (1-napthyl)-ethylenediamine dihydrochloride was added and the tubes were allowed to stand for 1 hour while the colour developed. Samples were then read at 560 mμ in a Beckman spectrophotometer against a reagent blank which had been carried through the diazotization procedure. Optical density values were converted to terms of mμMoles kynurenine by the use of a calibration curve which was frequently checked. In early experiments an aliquot of the 5% trichloroacetic acid extract was made up to 10 ml. with 5% trichloroacetic acid and on diazotization a conversion factor of OD_{560} x 0.779 mμMoles of kynurenine was obtained. In May and December, 1958, conversion factors of 0.45 and 0.514 x OD_{560} were obtained when the aliquot was diluted with water. The activities of the samples were corrected for endogenous production of kynurenine and dilution. Results are expressed as μMoles of kynurenine produced per hour per g. wet weight of liver or dry weight of liver. The values obtained where two volumes of liver suspension were assayed were averaged. Any alterations in this procedure are stated in the experimental section where they are applicable.

E. INDUCTION OF TRYPTOPHAN PYRROLASE IN VITRO

The activity of TP in liver slices can be increased by incubating the slices in a medium containing tryptophan (160,161). Details concerning the conditions necessary for this increased activity form part of the experimental part of this thesis so that only a bare outline is presented in this section.

After sacrifice by decapitation, the liver was removed immediately

from a rat. The liver was rinsed with cold water, blotted dry and chilled on ice. Blocks of liver were cut and sliced using a Stadie-Riggs microtome and blade. All instruments and glassware werekept chilled. Slices varied in weight between 50 and 150 mg. dependency on the top surface area of the block of liver sliced. They were of fairly uniform thickness. In a single experiment 36 slices of approximately the same size were divided into six groups of approximately equivalent weight. Each group was subject to a different treatment. Each slice was added to a 20 ml. beaker containing 2 to 4 ml. of Krebs-Ringer-Phosphate solution, pH 7.4 (204), in which tryptophan or other substances were dissolved. The beakers were then incubated for varying periods of time at 37° on the Dubnoff shaker. After the "induction period" the slices were rinsed free of the inducing medium by pouring the contents of the beakers into filter paper funnels and rinsing them four to five times with fresh aliquots of Krebs-Ringer-Phosphate solution. This procedure was carried out at 2°.

The slices (4 to 6) from each group were pooled and suspended in 0.14 M KCl containing 0.0025 N NaOH using a Teflon pestle and glass homogenizing tube. This suspension, omitting centrifugation, was assayed for TP activity as described under section D.

F. KYNURENINASE ASSAY

The procedure used for determining kynureninase activity was essentially the one used by Knox (189,202). The chief difference lies in the method employed for measuring the endproduct of kynureninase action on

kynurenine. Anthranilic acid was determined fluorometrically in the experiments to be described. Knox employed optical density measurements.

Rats were sacrificed by decapitation and the livers were immediately removed. The livers were rinsed in cold tap water, blotted dry and weighed. After mincing with scissors, the liver was suspended in 1 volume of ice-cold 0.14 M KCl containing 0.0025 N NaOH using a Teflon pestle and glass homogenizing tube. The suspension was centrifuged at 2° for 20 minutes at 35,000 x g. The supernatant was diluted to the original volume of the suspension by the addition of 0.02 ml. of 0.77 M $MgSO_4$/ml. of the original volume of suspension and 0.2 M K_2HPO_4-KH_2PO_4, pH 7.4. Reaction mixtures of 2 ml. contained 0.5 ml. of the liver supernatant preparation. To ensure saturation of kynureninase with its coenzyme, 20 to 120 µg. of pyridoxal phosphate were preincubated with the enzyme preparation for 15 to 30 minutes at 37° before the addition of the substrate or inhibitors. The reaction mixture contained 1.5 to 7.5 µMoles of L-kynurenine as substrate; the exact concentration is indicated in the context of the experimental results. The enzyme preparation was always incubated without the addition of kynurenine to determine the endogenous activity of the enzyme.

Incubation of the reaction mixtures were carried out in 20 ml. beakers at 370 in a Dubnoff shaker. The reaction was stopped by the addition of 5 ml. of 1% boric acid in ethanol. After centrifugation, the supernatants were diluted and their content of anthranilic acid determined by the fluorescence emission at 405 mµ (activation at 300 mµ) in the Amino-Bowmann spectrophotofluorometer. The units of fluorescence were converted to mµMoles of

anthranilic acid by employing a calibration curve. All samples were corrected for dilution and endogenous activity. In some cases kynurenine was determined by its absorbance at 360 mμ in a Beckman spectrophotometer however these results were not always reliable due to the presence of other interfering material.

III EXPERIMENTAL RESULTS

A. THE ROLE OF DIETARY TRYPTOPHAN IN SUPPORTING 5-HYDROXYINDOLE SYNTHESIS IN THE RAT

i) 5-Hydroxytryptamine

In preliminary experiments on the intestinal content of 5-HT of rats a value of 2.39 ± 0.43 μg./g. whole intestine was obtained from four rats. These rats were maintained on Purina Fox Chow pellets. This value is low in comparison to the values of Erspamer (134,135) who estimates that the gastro-intestinal tract of a rat contains 80 μg., Udenfriend and Weissbach who estimate that 10 to 20 μg. 5-HT/g. is found in the stomach and intestine of the rabbit (108), and Feldberg and Toh (124) who find 4 to 10 μg./g. intestine in the dog and rabbit.

In order to test the effect of dietary tryptophan on 5-HT intestinal levels in the rat, rats weighing 110 to 125 grams were randomly assigned to a diet containing acid-hydrolysed casein (Diet 4. described under section II Methods B ii) deficient in tryptophan or supplemented with 0.4% L-tryptophan. The rats were placed on the diet without a preliminary fasting period. Two rats were sacrificed after one day on the diet supplemented with tryptophan

and two were sacrificed from each dietary group on the fourth and eighth days of the dietary treatment. The intestines were removed from the stomach to the base of the colon and assayed as described under section II Methods C i. In this experiment all the rats although consuming a normal amount lost weight. It was noted that the diet had become rancid and this may have affected its nutritive value. The 5-HT estimated is summarized in Table VI_A

Table VI_A

The Effect of Dietary Tryptophan on Intestinal 5-Hydroxytryptamine Content

All values are expressed as µg. 5-HT/g. whole intestine. The entire intestinal tract was assayed as described under section II C i.

Days on diet	Tryptophan Deficient Diet[a]	Tryptophan Sufficient Diet[a]
1	-	1.38
	-	2.13
4	2.65	3.43
	1.01	2.70
8	1.18	2.94
	2.58	1.93

a See section II Methods B ii for composition of Diet 4. L-tryptophan was added to one diet.

The results of this experiment were not clear-cut. After one day on the complete diet the intestinal 5-HT tended to be lower than the four control values achieved in preliminary experiments (2.39 ± 0.43 µg. 5-HT/ g. whole intestine). On the fourth and eighth days one deficient rat had a definitely lower 5-HT content but the other was decidedly within the control range.

Of the tryptophan-sufficient rats, one sacrificed on the eighth day had a decreased 5-HT intestinal content.

A second experiment was carried out to see whether with a fresh diet there would be an unambiguous effect of tryptophan deficiency. Rats weighing between 160 and 228 g. were randomly assigned to diets deficient in or supplemented with DL-tryptophan. These rats were fasted for twenty-four hours prior to placing them on the diets. Only the small intestine was assayed for its content of 5-HT. The method used is as described before in II Methods, C i. The results are summarized in Table VI_B.

Table VI_B

The Effect of Dietary Tryptophan on Intestinal 5-Hydroxytryptamine Content

All values are expressed as µg. 5-HT/g. small intestine. Only the small intestine was assayed as described under section II Methods C i.

Days on Diet	Tryptophan Deficient Diet	Tryptophan Sufficient Diet
1[a]	2.55	6.95
	2.62	3.55
7[b]	3.13	6.05
	3.68	7.16
	4.78	6.60
	3.69	6.53
14[a]	1.39	4.17
	2.46	-

a Diet containing zein deficient in or supplemented with 0.4% DL-tryptophan as described under II Methods B ii, Diet 6.

b Diet containing zein deficient in or supplemented with 1% DL-tryptophan as described under II Methods B ii, Diet 5.

This experiment demonstrates clearly the detrimental effect of tryptophan deficiency on the intestinal content of 5-HT. A dietary tryptophan deficiency is manifested by a marked decrease in 5-HT within one day as can be seen from the data of Table VI_B.

Eber and Lembeck (105) found values for the 5-HT content of the small intestine of rats similar to the values in Table VI_A for the whole intestine. In dietary deficiency studies similar to these they found a significant decrease in the 5-HT content of the small intestine after two weeks in one experiment and only after a month in a second experiment. The method used by these investigators was bioassay on the rat uterus.

ii) 5-Hydroxyindoleacetic Acid

Dietary deficiency experiments were also carried out to determine the effect of tryptophan deficiency on the urinary excretion of 5-HIAA. This metabolite accounts for approximately one third of the endogenously metabolized 5-HT in the rat (134,135) and the dog (28). On this basis, urinary 5-HIAA gives a measure of the turnover of 5-HT in the rat and, in particular, may reflect the availibility of its precursor, tryptophan.

In order to compensate for variable food consumption rats were randomly assigned to their respective diets and subjected to a preliminary twenty-four hour fast. Four rats were placed on tryptophan-deficient and tryptophan-sufficient diets, respectively, the urine of four rats being pooled to ensure an adequate urine volume. Urines were collected as described under II Methods C ii for a twenty-four hour period. 5-HIAA was determined as

described under this same section. Table VII summarizes these results. The values presented for the fourth day represent those obtained from a separate group of rats; although higher values, they present the same relation to diet composition as the others.

Table VII

The Effect of Dietary Tryptophan on the Excretion of 5-Hydroxyindoleacetic Acid by the Rat

Urinary 5-HIAA was collected and assayed as described under section II Methods C ii.

Days on Diet	Tryptophan Deficient µg. 5-HIAA/100 g./24 hrs.	Tryptophan Sufficient µg. 5-HIAA/100 g./24 hrs.
1[a]	5.10	10.81
4[b]	12.79	21.78
5[a]	7.96	15.77

a These rats weighed 150 to 170 g. before being placed on Diet 6 described under section II Methods B ii with or without added tryptophan.
b These rats weighed 170 to 228 g. before being placed on Diet 5 described under section II Methods B ii with or without added tryptophan.

These results show that even after one day on a diet deficient in tryptophan, the urinary excretion of 5-HIAA is reduced by 50%. This order of difference is maintained for five days which indicates that the major adjustment to this deficiency was made within the first day. The intestinal content of 5-HT was also shown to be markedly diminished within one day (Table VI_B). This difference can be explained in two ways: (1) the preliminary fasting period reduced all the 5-HT depots to basal metabolic levels which were

maintained through an endogenous tryptophan supply in the deficient rats and increased in excess of the endogenous levels by dietary tryptophan in the tryptophan-fed rats; or (2) the deficient rats suffer a decline in their 5-HT stores as is suggested in Table VI_B.

Erspamer's estimate of the urinary excretion of 8.14 µg. 5-HIAA/100 g./24 hrs (134,135) by the rat agrees with these figures, more particularly with the figures from the rats given no tryptophan supplement.

iii) The Effect of Reserpine and Dietary Tryptophan on 5-Hydroxyindoleacetic Acid Excretion in Rats.

Another aspect of this problem was to determine whether treatment with reserpine would influence the urinary excretion of 5-HIAA in rats fed diets containing tryptophan or deficient in the amino acid. Reserpine is known to deplete the body depots of 5-HT (138,139,205) and to interfere with the 5-HT binding sites (145,146). Erspamer and Ciceri (144) found that, in spite of the continuous administration of reserpine to rats, after an initial increase in urinary 5-HIAA the daily excretion persists at normal levels.

In order to test the sensitivity of the 5-hydroxyindole synthesizing system to tryptophan supplied by the diet, two injections of reserpine were given to deplete the body stores of 5-HT. Three dietary treatments were tested for their effect on the maintenance of endogenous 5-HT stores. One diet was supplemented with D-tryptophan in order to test the efficacy with which it is utilized. A second diet was supplemented with L-tryptophan to serve as a control. A third diet was deficient in tryptophan in order

to test the ability of endogenous tryptophan alone to maintain 5-HT production. Two experiments were performed. In each half the animals were treated with reserpine, half with placebo. In the first experiment (Table VII) rats weighing 106 to 116 grams were randomly assigned to the three diets. On the third and sixth days of the diet half of the animals received 2 mg. reserpine/kg. intravenously in the tail vein, half received placebo. Urines were collected for periods of six to sixteen hours on the days indicated in Table VIII and assayed for their 5-HIAA content as described under II Methods C ii. The rats receiving reserpine exhibited the typical symptoms observed by others (205,206,207) of sedation, ptosis of the eyelid and diarrhea. Many had bloody paws and noses and all lost over 10% of their body weight in the first twenty-four hours following reserpine. One animal which received reserpine assumed a hunchback position, although on the diet containing tryptophan; this position was maintained for the duration of the experiment.

Table VIII*

Urinary Excretion of 5-Hydroxyindoleacetic Acid in the Rat: as Influenced by (1) Prior Treatment With Reserpine, and (2) Tryptophan Feeding

5-HIAA is expressed as µg./10 hrs./kg. and each sample represents the amount excreted by three rats. The assay of 5-HIAA is described under section II Methods C ii. The intravenous injection of 2 mg./kg. of reserpine (R) or placebo was given on the third and sixth days of the experimental dietary regime.

Days on Diet	Days after R dose		Urinary 5-HIAA					
			Placebo			Reserpine		
	1st	2nd	Nil[a]	D-[b]	L-[c]	Nil[a]	D-[b]	L-[c]
7	4	1	45	24	65	91	74	138
10	7	4	-#	138	171	-#	87	103
12	9	6	11	189	249	49	167	-#
17	14	11	10	-#	81	18	24	69
21	18	15	75	80	133	17	84	70
Mean excretion of 5-HIAA			35	108	140	44	87	95

a Rats were fed the basal Diet 3 described under section II Methods B ii.
b Rat Diet 3 contained 0.2% D-tryptophan.
c Rat Diet 3 contained 0.2% L-tryptophan.
* This experiment was carried out with the assistance of J. Katis, a medical student, employed in our laboratory during the summer, 1957.
# Sample lost.

The overall picture presented in Table VIII is one of a gradual depletion of 5-hydroxyindole stores in the tryptophan-deficient rats treated with reserpine. Deficient rats treated with placebo, although showing a variable excretion of 5-HIAA. also showed a marked diminution in this constituent. Except for the single value on the twenty-first day these rats excreted less than the

reserpine-treated rats. This higher excretion by the deficient rats treated with reserpine might be explained in terms of the decreased amine binding in the tissues of these rats. In this way the "overflow" of 5-HT synthesized from endogenous tryptophan is more readily available for oxidation by monoamine oxidase and aldehyde oxidase to 5-HIAA. Both the D- and L-isomers of tryptophan appear to be equally efficient in supporting the synthesis of 5-hydroxyindoles. This indicates that the rat can convert D- to L-tryptophan efficiently for 5-hydroxyindole synthesis. The mechanism of this conversion is not known but it may proceed via D-amino acid oxidase to 5-hydroxyindole-pyruvic acid and via transamination of the latter to L-5-hydroxytryptophan.

It was considered that by extending the period for urine collection less variable results due to diurnal variation and due to the error involved in working with small urine volumes (particularly from the rats treated with reserpine and fed diets deficient in tryptophan) might be obtained. Twenty-four rats weighing between 86 and 102 grams were randomly assigned to basal diets containing hydrolysed casein. Two of the basal diets contained 0.4% D- or L-tryptophan, the third did not contain tryptophan. Erspamer and Ciceri (144) found doses of 0.5 and 1 mg. reserpine/kg. were lethal to the majority of their rats after several days of treatment. In the previous experiment 2 mg./kg. administered on two occasions to our rats resulted in sick animals the majority of which never appeared normal for the duration of the experiment. It was decided to decrease the dosage of reserpine. Half the animals were injected intravenously in the tail vein with reserpine (1 mg./kg.) and half with placebo on the fifth and twelfth days on the experimental diets. The

same sedative action and diarrhea was observed in this experiment as in the previous one. The results are summarized in Table IX.

Table IX

Urinary Excretion of 5-Hydroxyindoleacetic Acid in the Rat: as Influenced by (1) Prior Treatment with Reserpine and (2) Tryptophan Feeding

5-HIAA is expressed as µg./10 hrs./kg. and each sample represents the excretion from four rats. The assay of 5-HIAA is described under section II Methods C ii. The intravenous injection of 1 mg./kg. of reserpine (R) or placebo was given on the fifth and twelfth days of the experimental dietary regime.

Days on Diet	Days after R dose		Urinary 5-HIAA					
			Placebo			Reserpine		
	1st	2nd	Nil[a]	D-[b]	L-[c]	Nil[a]	D-[b]	L-[c]
3	-	-	51	116	103	59	101	94
7	2	-	29	117	98	27	104	71
10	5	-	18	68	65	23	78	31
14	9	2	15	60	51	24	45	51
17	12	5	37	61	52	41	79	65
Mean excretion of 5-HIAA			30	84	74	35	81	62

a Rats were fed the basal Diet 4 described under section II Methods B ii.
b Rat Diet 4 contained 0.4% D-tryptophan.
c Rat Diet 4 contained 0.4% L-tryptophan.

A 50% reduction in the urinary 5-HIAA excretion can be seen in Table IX to have occurred on the third day of the dietary regime where tryptophan was not contained in the diet. This finding supports the observation of Table VI_B where a 50% reduction in intestinal 5-HT stores was found after one day on a diet deficient in tryptophan. In the placebo-treated rats the urinary 5-HIAA

appeared to gradually decline with the exception of the value obtained on the seventeenth day of the diet. On the tenth and fourteenth days the values were reduced by at least a third; this reduction is obscured by apparent decreases in the 5-HIAA excretion by rats fed tryptophan. Both the D- and L-isomers of tryptophan were found to be equally efficient in maintaining 5-hydroxyindole synthesis, confirming the results of the previous experiment. No increase in 5-HIAA excretion was observed on the second day after reserpine treatment in either the tryptophan deficient- or supplemented-rats. Reserpine does not appear to modify the excretion of 5-HIAA from rats fed diets containing tryptophan or from which tryptophan was omitted. These results support the findings of Erspamer and Ciceri (144) and Brodie et al. (145) that reserpine does not affect the biosynthesis of 5-HT. The diminution of 5-HIAA excretion observed on the first day in rats maintained on tryptophan-deficient diets (Table VII) is found to be maintained fairly constantly for a period of three weeks (Tables VIII and IX) demonstrating a certain metabolic adjustment to the reduced dietary tryptophan supply. This is seen in the levels of 5-HIAA excreted after reserpine treatment by the deficient rats.

B. THE INFLUENCE OF THIAMINE AND TRYPTOPHAN ON THE TRYPTOPHAN PYRROLASE ACTIVITY OF RAT LIVER

The experimental data from which Dalgliesh (180,181) deduced a role for thiamine at the TP level of tryptophan metabolism were followed up in an attempt to define this role more sharply. Experiments in vivo and in vitro are presented in which thiamine has been tested for its effect on the

constitutive levels and adapted formation of TP. These experiments include one in which the antimetabolites oxythiamine and pyrithiamine were administered to rats and their effects on the constitutive and adapted levels of TP determined. Furuta has found that administration of oxythiamine to rats reduces the TP activity of the liver (208). Experiments in which oxythiamine and pyrithiamine have been tested for their effect on the induction of TP in vitro are presented in the following section C. The effect of a dietary tryptophan deficiency on the constitutive level of TP has also been studied. The dietary L-tryptophan constituent was replaced by the D-isomer in two experiments in order to determine the efficacy with which D-tryptophan supports the constitutive level of TP.

i) Acute Experiments

In order to see whether thiamine might directly influence the activity of TP thiamine was injected subcutaneously into rats and the liver activities determined. The injections of 2 mg. thiamine/rat and 0.25 mM of L-tryptophan in saline were made 3 hours before the rats were sacrificed. Two control animals were injected with isotonic saline and L-tryptophan respectively. The relative TP activities of the four animals were:

control rat	100%	injected with L-tryptophan	352%
injected with thiamine	96%	injected with both	173%

These results indicate the thiamine does not elicit an adaptive response in TP nor does it stimulate the response due to the injection of tryptophan.

The possibility presented itself that thiamine may activate the

TP system when it is added as diphosphothiamine (DPT) to the system *in vitro*. Tryptophan is known (39,155) to protect the labile enzyme system *in vitro* and so DPT was tested for possible stabilizing action on TP. The results of these experiments are summarized in Table X. TP activity was assayed as described under section II Methods D with the modifications indicated in the footnote to Table X.

Table X

Effect of Diphosphothiamine (DPT) on Tryptophan Pyrrolase (TP) *in vitro*

TP activity is expressed as μM. of kynurenine formed/hr./g, liver. Reaction mixture 1, described under section II D, was employed in these assays.

Expt.	Addition	TP activity
A[a]	Tryptophan, was added to enzyme at zero time	1.11
	DPT, added at 0 min.	0.01
	Both, added at 0 min.	1.06
	Tryptophan, added after enzyme had been incubated 30 min. at 37°	0.40
	DPT, added at 30 min.	0.00
	Both, added at 30 min.	0.40
B[b]	Dialysed liver extract[c]	
	Tryptophan	0.76
	Tryptophan - DPT	0.46
	Non-dialysed liver extract[c]	
	Tryptophan	2.73
	Tryptophan - DPT	2.28

a The enzyme was contained in the supernatant fraction of a 25% isotonic KCl rat liver homogenate.

b The enzyme was contained in the supernatant fraction of a 20% isotonic KCl rat liver homogenate.

c The supernatant fractions in Expt. B were either dialysed against 0.0002 M L-tryptophan in isotonic KCl or made up to that concentration and held for 22 hrs. at 2° C. The data were corrected for kynurenine formed during this period.

These results indicate that DPT neither activates nor stabilizes the TP enzyme system *in vitro*. In Expt. A it is seen that DPT had no effect on the oxidation of tryptophan nor a reactivating effect on the aged preparation. Dialysis of the enzyme preparation, as is indicated in Expt. B, results in considerable loss of the activity even in the presence of tryptophan. The addition of DPT to this assay system did not stimulate the activity. An extensive dialysis of the liver supernatant fraction against 0.9% KCl containing 0.6% Versene completely inactivated the enzyme. The addition of magnesium ions or DPT or both to this preparation were without effect.

ii) The Effect of Thiamine and Tryptophan Deficiencies on Constitutive Tryptophan Pyrrolase Activity.

Because no direct effect of thiamine or thiamine pyrophosphate could be demonstrated on the activity of TP *in vitro* the possibility of an indirect action of this vitamin was investigated. A multifactorial dietary experiment was conceived in which the influence of thiamine or interaction of thiamine with tryptophan could be deduced from the resultant activities of the liver TP. The experiment was replicated on two separate occasions and the results of the two experiments were subjected to statistical analysis.

In two experiments rats weighing 82 to 98 g. and 110 to 130 g. respectively were randomly assigned to five diets. A total of six rats received each diet. Diet 1 was used in one experiment, Diet 2 in the second (see section II Methods B ii). Purina chow was used as one diet to provide a control for the fully supplemented synthetic diet. The experimental diets

were varied in the following way:

Diet I. Diet 1 or 2 supplemented with 0.9% DL-tryptophan and thiamine.

Diet II. Purina chow pellets, powdered.

Diet III. Diet 1 or 2 supplemented with 0.9% DL-tryptophan but deficient in thiamine.

Diet IV. Diet 1 or 2 supplemented with thiamine but deficient in tryptophan.

Diet V. Diet 1 or 2 with both tryptophan and thiamine omitted.

The animals receiving Purina chow grew faster than those receiving the fully supplemented synthetic diets, although the latter gained weight steadily throughout the dietary period. Fig. 2 exemplifies the growth of rats on Diet 2.

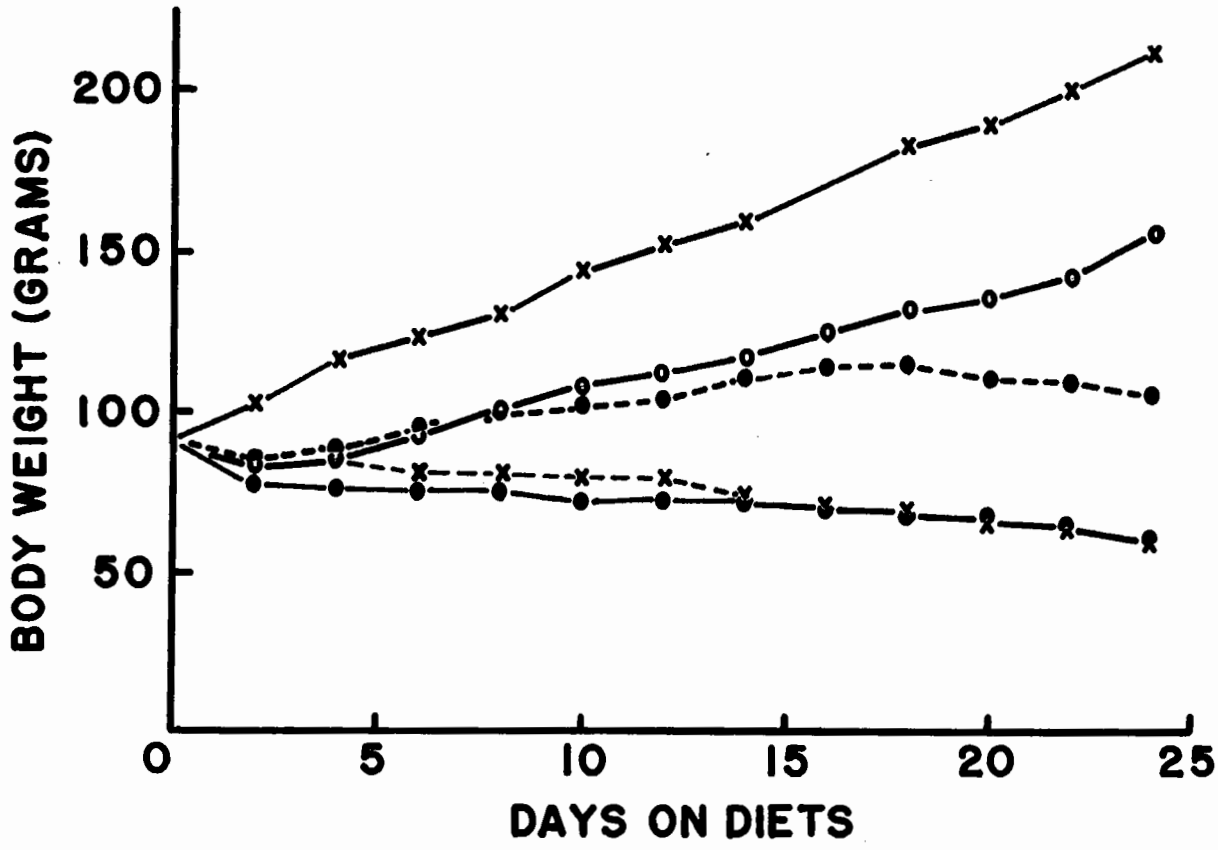

Figure 2. Growth curves of rats on experimental diets. Each point represents the mean weight of three animals (to 12th day) or two animals (after the 12th day).

×——× rats fed Purina chow;
○——○ rats fed diets supplemented with tryptophan and thiamine;
●- - -● rats fed diets deficient in thiamine;
×- - -× rats fed diets deficient in thiamine and tryptophan;
●——● rats fed diets deficient in tryptophan.

At least two factors could account for the differences in weight gain of the animals on Diets I and II. The casein hydrolysate may not have contained the requisite amount of the aromatic amino acids phenylalanine and tyrosine to support optimum growth and secondly, the rats consuming Diet II engested far more than those fed Diet I. Palatibility may have contributed to the second factor because Diet I was of a very greasy consistency. Rats fed diets deficient in tryptophan ceased growing almost at once while the rats fed diets deficient in thiamine only exhibited the effects of avitaminosis at about the fourteenth day.

The six sets of rats (i.e. one animal from each dietary treatment) were sacrificed after varying periods of time on the dietary regime and their livers were assayed for their TP activity. The assay was carried out as described under section II Methods D. In this way data were obtained on the 12th, 13th (two sets), 14th, 24th and 28th days of the dietary regime. These data as well as two other parameters, change in body weight and liver weight, are presented in Table XI.

Table XI

Effect of Thiamine and Tryptophan Deficiencies on Body Weight, Liver Weight and Tryptophan Pyrrolase of Rat Liver

Change in body weight is the difference between the initial weight at the beginning of the dietary regime and the final weight. Liver weight is the weight at the time of sacrifice. TP was assayed as described under section II Methods D (reaction mixture 1): the enzyme was contained in the supernatant traction of a 50% rat liver homogenate except in one assay where the enzyme was contained in the supernatant of a 20% rat liver homogenate. Activity is expressed as μMoles of kynurenine/hr./g. liver.

Dietary Supplement*	Period of time on Dietary Regime (Days)					
	12	13	13	14	24	28
	Change in Body Weight, g.					
Thiamine plus tryptophan ("Complete")	27	26	24	28	67	69
Tryptophan only	32	6	21	14	15	18
Thiamine only	-29	-24	-20	-31	-24	-35
None	-24	-29	- 8	-31	-27	-35
Purina chow diet	59	77	72	78	147	130
	Liver Weight, g.					
Thiamine plus tryptophan ("Complete")	7.13	6.47	6.09	8.34	8.63	8.10
Tryptophan only	7.10	6.30	5.44	6.09	4.15	4.42
Thiamine only	3.12	3.06	3.49	3.42	3.33	2.77
None	3.49	3.33	3.24	3.87	2.79	2.95
Purina chow diet	7.15	8.77	7.18	9.32	10.37	9.84
	TP activity					
Thiamine plus tryptophan ("Complete")	1.72	2.04	3.35	1.64	3.05	6.02
Tryptophan only	2.67	1.54	3.97	1.77	3.69	3.09
Thiamine only	0.86	1.58	0.80	1.66	0.89	5.62
None	0.62	1.01	1.65	1.28	0.68	2.57
Purina chow diet	1.07	1.04	2.45	1.46	2.50	3.45

*Diets are described under section II Methods B ii Diets 1 and 2.

The data of Table XI show that the experimental diets led to marked differences in body weight, liver weight and TP activity. In order to account for any effect independent of the quality of the diets, which such changes may have had on the TP activity, an analysis of covariance was carried out as described by Snedecor (209). The results of this analysis (Table XII) indicate that the differences in TP activity as a result of the different diets were statistically significant.

Table XII

Analysis of Covariance of Data on Change in Body Weight, Liver Weight and Tryptophan Pyrrolase Activity of Liver

Sources of variation	Degrees of freedom	Mean square	F ratio
All	29		
Diets	4	4.347,845	11.762*
Replicates	5	5.503,981	14.889*
Remainder	20	0.475,893	-
Regression of tryptophan pyrrolase activity on body weight change and liver weight	2	1.432,029	3.874**
Error	18	0.369,656	(1.0)

*Highly significant (P less than 0.01).
**Significant (P less than 0.05).
This variance has been employed to calculate the difference necessary to establish significance between any two mean values of tryptophan pyrrolase activity each based upon six rats, as given in Table XIII ("Adjusted Means"). The formula used is

$$\text{Necessary difference} = t\sqrt{(2 \times \text{mean square})/6}.$$

For 2(n-1)=10 degrees of freedom, and P=0.05, =2.228. Hence the minimal difference necessary for significance = 0.78 μM. kynurenine/hr./g. liver.

Because the regression of TP activity on liver weight and change in body weight was significant, it was possible to adjust statistically (209) the mean values of TP activity on each of the diets in order to exclude the effect of these two independent variables. (I am indepted to Dr. T.L. Sourkes for performing this statistical adjustment). The adjusted mean values are given in Table XIII where it is seen that the adjustments are not very marked in spite of the significant regression.

Table XIII

Effect of Thiamine and Tryptophan Deficiencies on Body Weight, Liver Weight, and Tryptophan Pyrrolase Activity of the Liver

Supplement	Change in body weight, g.	Liver weight, g.	TP activity (μM. kynurenine/hr./g. liver)	
			Observed	Adjusted
Thiamine plus tryptophan ("Complete")	40.2	7.5	2.97	3.04
Tryptophan only	17.7	5.6	3.21	3.22
Thiamine only	-27.2	3.2	1.39	1.42
None	-25.7	3.3	1.44	1.47
Purina chow diet	93.8	8.8	1.94	1.81

Each figure is a mean of the values obtained from six rats (see Table XI). Adjusted values of TP activity were calculated by use of the respective regression coefficients of liver weight and body weight change on TP activity, estimated by standard statistical procedure (209). This calculation was made by Dr. T.L. Sourkes.

The difference necessary for significance at the 5% level of probability, calculated from the residual variance in Table XII is 0.78 μM. of kynurenine/hr./g. liver. Of the TP values in Table XIII only those obtained from rats fed the synthetic diet supplemented with tryptophan are significantly higher than the other three. The omission of thiamine from the diet did not affect the activity of TP and no interaction with tryptophan can be deduced from these results. The TP activity of rats fed Purina chow was significantly lower than the activity of rats fed synthetic diets supplemented with tryptophan while it was not significantly different from the activity of rats fed diets deficient in tryptophan. Three explanations may clarify this effect: firstly, the synthetic diets contained 0.9% tryptophan. Rose has estimated that 0.2% is necessary for the growth of rats (9). The high levels of TP activity observed with the tryptophan supplement may thus represent increased constitutive levels as a result of induction or stabilization of the enzyme systems *in vivo*. The tryptophan content of Purina chow was not determined but it may not contain such a high concentration of tryptophan. Secondly, because the rats were not adrenalectomized the stressing effect of tryptophan deficiency may have stimulated a hormonal induction of the TP system so that these values may not be truly representative of a "basal constitutive level". Lastly, Purina chow probably offers the amino acids as protein, in which form they may have been differentially absorbed.

iii) The Effect of D- and L-tryptophan on Constitutive Tryptophan Pyrrolase Activity.

The effect of D- and L-tryptophan on the constitutive level of TP was studied in two dietary experiments. In each experiment diets were made up to contain D-tryptophan, L-tryptophan and no tryptophan, respectively. Where tryptophan was a constituent it made up 0.2% in one replicate and 0.4% in the second replicate of the diets. The composition of the diets is described under section II Methods B ii: Diet 3 was used in one experiment, Diet 4 in the second. In each experiment half the animals from each dietary group were injected with reserpine and half with placebo, the solvent used to solubilize the alkaloid, as part of the study described under section III A iii on urinary 5-HIAA excretion (Tables VIII and IX).

Twenty-four animals weighing between 86 and 102 g. and eighteen weighing between 106 and 116 g. were randomly assigned to the three diets in each experiment. The animals were maintained on the dietary regime for 21 to 25 days after which they were sacrificed and their livers assayed for TP activity. Three parameters were determined, body weight, liver weight and TP activity, and these data are presented in Table XIV.

Table XIV

Effect of D- and L-tryptophan and Reserpine on Body Weight, Liver Weight and Tryptophan Pyrrolase Activity

Change in body weight represents the difference between the initial and final weights of the rats on the experimental diets. Liver weight was taken at the time of sacrifice. TP activity is expressed as μM. of kynurenine formed/hr./g. liver. The liver was assayed for TP activity by the method described under section II Methods D. The enzyme was contained in the supernatant fraction of a 20% rat liver homogenate and was assayed in reaction mixture 1. Reserpine was injected intravenously (2 mg./kg. in one experiment, 1 mg./kg. in a second) on two occasions to half the animals. Control rats received placebo.

Reserpine	Supplement	Days of Treatment 21				22	24	25
		change in body weight, g.						
-	Nil	-29[a]	-20[b]	-15[b]	-19[b]	-19[b]	-16[a]	-14[a]
-	D-	-20	- 2	- 6	-10	-10	+15	+13
-	L-	-14	-13	-12	- 3	-11	+12	+ 8
+	Nil	-42	-15	-23	-16	-19	-23	-23
+	D-	-14	+ 1	- 8	- 6	- 2	+ 2	+10
+	L-	- 4	-12	- 7	-14	- 4	- 4	+10
		liver weight, g.						
-	Nil	2.81	3.83	2.59	2.55	2.63	2.60	3.69
-	D-	2.90	3.42	2.86	3.60	3.31	4.30	5.40
-	L-	2.60	4.67	3.67	3.82	2.54	4.74	4.66
+	Nil	2.51	2.92	2.33	2.99	2.53	3.11	4.03
+	D-	2.64	3.13	2.90	-	3.15	3.82	4.53
+	L-	3.31	3.15	2.54	2.68	3.07	4.37	4.94
		TP activity						
-	Nil	1.33	2.23	2.62	2.38	3.55	1.82	3.28
-	D-	4.42	5.25	5.59	4.84	8.12	2.33	-
-	L-	7.77	5.73	8.11	4.84	8.52	2.61	4.29
+	Nil	2.42	3.05	3.79	1.83	3.68	1.28	1.69
+	D-	4.31	4.40	9.80	-	9.67	1.94	9.40
+	L-	3.18	4.35	6.23	7.52	5.84	1.87	9.27

a Rats fed Diet 3 described under section II Methods B ii: 0.2% D- or L-tryptophan was added as indicated.

b Rats fed Diet 4 described under section II Methods B ii: 0.4% D- or L-tryptophan was added as indicated.

These data were subjected to statistical evaluation (209).

Sources of Variation	Degrees of Freedom	Mean Squares: Change in body wt.	Liver wt.	TP activity
All	41[a] 2[b]			
Missing data	1			
Between diets	5	569.92	0.95	21.13
Between replicates	6	286.74	2.54	14.33
Remainder, for error determination	29[a] 28[b]	46.94	0.21	2.47

[a] Rat died
[b] Sample lost (TP).

The effects of reserpine and tryptophan are summarized in Table XV.

Table XV

Effect of Reserpine, D-tryptophan and L-tryptophan on Rat Liver Tryptophan Pyrrolase

These data represent a summary of the results compiled in Table XIV. For details consult the addenda to Table XIV.

Reserpine	Supplement	No. of rats	Change in body wt., g.	Liver weight, g.	TP activity
-	None	7	-18.8	2.9	2.46
	D-Tryptophan	7	- 2.8	3.7	5.09
	L-Tryptophan	7	- 4.7	3.8	5.98
+	None	7	-23.0	2.9	2.53
	D-Tryptophan	6	- 2.4	3.4	6.59
	L-Tryptophan	7	- 5.0	3.4	5.47
Standard deviation of means* (a) Of 6 rats			± 2.8	±0.19	± 0.64
(b) Of 7 rats			± 2.6	±0.17	±0.59

*Calculated from the mean square for error obtained in the analysis of variance, after removal of variance between the diets and between the replicates.

The analyses of the data obtained in this experiment (Table XV) show that reserpine has no effect on TP activity. On the other hand the rats receiving the tryptophan-free diet showed statistically significant decreases (with a probability of 0.05 or less) in their body weight, liver weight and TP activity. The D-isomer of tryptophan was demonstrated to be equally as efficient as the L-isomer for the maintenance of growth, liver weight and TP activity. The basic data of Table XIV show that even the rats receiving tryptophan in many cases suffered weight losses on the dietary regime. A major factor contributing to this loss is undoubtedly the frequent fasting periods imposed on the rats at intervals throughout the dietary regime for the purpose of collecting urine for 5-HIAA estimation. In each replicate of this experiment five such fasting periods were imposed on the rats. The low levels of TP found in the livers of rats fed the tryptophan-deficient diets probably represents the activity due to endogenous tryptophan and hormonal influences.

iv) The Effect of Thiamine Deficiency and Thiamine Antimetabolites on Tryptophan Pyrrolase Induction *in vivo*.

It was felt that since no effect of thiamine deficiency was demonstrated on the constitutive level of TP it might still affect the increase in activity of this enzyme following tryptophan administration. Two experiments were designed to investigate this possibility. In one experiment rats were placed on diets with and without thiamine. In the second experiment all the rats were fed a basal diet from which thiamine was omitted. The thiamine analogues, oxythiamine and pyrithiamine, were administered daily with thiamine

to rats in this experiment. Wolfe (210) found that the simultaneous administration of thiamine with these antimetabolites considerably reduced the loss in weight due to oxythiamine administration but was without effect on the rapid weight loss and paralysis which ensues with pyrithiamine administration. The simultaneous administration of thiamine does not obviate the inhibition of erythrocyte transketolation due to oxythiamine. On this basis it was felt that if thiamine were involved in the formation of this enzyme in vivo the administration of these potent analogues of thiamine would surely modify the activity of TP also.

In the study of simple thiamine deficiency on the activity of liver TP, ten rats weighing between 102 and 115 grams were randomly assigned to diets containing thiamine or from which thiamine was omitted. After 21 and 23 days the rats fed thiamine in their diets had gained from 74 to 95 grams: of the rats fed diets from which thiamine had been omitted, two gained 1 gram, one gained 4 grams and two lost 6 and 11 grams respectively.

In the study of the thiamine analogues eight rats received a basal thiamine-deficient diet. These rats, weighing between 108 and 129 grams at the start of the experiment, were randomly assigned to four treatments: (1) two rats received an intraperitoneal injection of 1 ml. of 0.9% saline daily, (2) two received 25 µg. of thiamine in 1 ml. of saline daily, (3) two received 100 µg. of oxythiamine and 25 µg. of thiamine in 1 ml. of saline daily and (4) two received 350 µg. of pyrithiamine and 25 µg. of thiamine in 1 ml. of saline daily. After thirteen days on this regime one rat receiving pyrithiamine had developed severe neuritic symptoms and one rat receiving

the thiamine-deficient diet had died. The following weight changes were noted:

thiamine	+ 68	+ 65 grams
thiamine and oxythiamine	+ 45	+ 48
thiamine and pyrithiamine	+ 5	+ 4
none	+ 28	+ 12 (10 days)

These weight changes amplify the severe deterioration noted of the rats receiving pyrithiamine.

In both experiments half the rats were given an intraperitoneal injection of tryptophan in saline before sacrifice to increase the activity of the liver TP. The remaining animals were given an equivalent volume of saline. The liver TP activity was assayed according to the method described under section II Methods D. The data obtained from the two experiments are presented in Table XVI.

Table XVI

Effect of Thiamine and Thiamine Analogues (Oxythiamine and Pyrithiamine) on Tryptophan Pyrrolase Induction with Tryptophan

Rats in Experiment A were fed basal diet 8 for 11 days and were then transferred to basal diet 7. Thiamine was added to these diets as indicated in the table. Rats in Experiment B were fed basal diet 7. Composition of the diets is described under section II Methods B ii. These rats received daily intraperitoneal injections of thiamine (25 µg), pyrithiamine (350 µg.) and oxythiamine (100 µg.) in saline as indicated in the table. Control rats received saline. TP activity is expressed as µMoles kynurenine formed/hr./g. liver. The enzyme was contained in the supernatant fraction of a 20% rat liver homogenate in Experiment A and in a 12.5% rat liver homogenate in Experiment B. Reaction mixture 1 was used in Experiment A, reaction mixture 2 in Experiment B. The assay of TP was carried out as described under section II Methods D. The induction period is the time interval between the administration of saline or tryptophan (inducer) and sacrifice of the animal; saline or tryptophan were injected intraperitoneally.

Expt.	Thiamine	Days on diet	Inducer	Induction period hrs.	TP activity
A	-	21	saline	4	2.74
	-	21	tryptophan[a]	4	10.09
	+	21	saline	4	1.40
	+	21	tryptophan[a]	4	12.70
	-	23	saline	4	4.34
	-	23	tryptophan[b]	4	22.46
	-	23	tryptophan[b]	4	27.11
	+	23	saline	4	1.80
	+	23	tryptophan[b]	4	15.59
	+	23	tryptophan[b]	4	18.93
B	-	13	tryptophan[b]	4.5	14.19
	+	13	saline	4.5	1.39
	+	13	tryptophan[b]	4.5	17.44
	+ plus pyrithiamine	13	saline	4.5	2.62
	+ " "	13	tryptophan[b]	4.5	26.18
	+ plus oxythiamine	13	saline	4.5	2.65
	+ " "	13	tryptophan[b]	4.5	12.53

a Rats received 0.46 mMoles DL-tryptophan/100 g. body wt.

b Rats received 0.50 mMoles DL-tryptophan/100 g. body wt.

The data of Table XVI indicate that thiamine deficiency does not affect the increase in TP activity following the administration of tryptophan. The deficient animals and those which received the thiamine antimetabolites, oxythiamine and pyrithiamine, showed increased constitutive levels of TP which can probably be attributed to a hormonal stimulation of the enzyme by these stressing agents. The animal which received pyrithiamine had an induced level of TP higher than that of the control rat following tryptophan administration. This suggests that two mechanisms were responsible for the adaptation in this animal; that of the response to exogenously administered tryptophan and additionally, hormonal stimulation. Since only one animal was tested for the effect of each treatment this deduction must be provisional. Schor and Frieden (158) found however a similar response with insulin and tryptophan. The administration of oxythiamine to a rat resulted in a decreased response to the tryptophan injection but nevertheless a considerable increase in the TP activity over the constitutive level was still achieved (2.65 to 12.53 μMoles kynurenine/hr./g. liver). Furuta found that the administration of oxythiamine to rats decreases the activity of TP following induction with tryptophan (208). The effect of oxythiamine in this experiment (Table XVI) was not felt to be striking enough to warrant further investigation by this means.

v) The Effect of Thiamine on Tryptophan Pyrrolase in Adrenalectomized Rats

It was felt that the study of the effect of thiamine on TP activity would not be complete unless it was studied in the absence of hormonal influence

from the adrenal glands. The administration of tyrosine, histamine, and histidine all increase the activity of TP (156,164,165) and they are considered in this respect to act through stimulation of the adrenal cortex (164,165). Other stressful agents such as irradiation (168) have also been found to mediate this effect through hormonal stimulation.

To this end two experiments were performed in which adrenalectomized and sham-operated rats were fed thiamine-deficient diets to determine the effect of these treatments on the adaptibility of TP. In the first experiment twenty rats were selected. The operations took place over three days. I am indebted to Dr. R.A. Cleghorn for performing many of the operations in the first experiment and for his instructions in this performance. The rats were anaesthetized with aldehyde-free ether: sixteen were adrenalectomized, four were sham-operated. One of the adrenalectomized rats died after the operation. Two to five days after the operations all the rats were placed on the dietary regime: half received the diet with thiamine, half without thiamine. At this time the rats weighed 125 to 174 grams and were randomly assigned to the diets from 'high' and 'low' weight groups. In view of the internal evidence in the first experiment which suggested that some adrenal regeneration had occurred by the time the TP assays were performed, the chronology was changed in the second experiment. In order to reduce the time interval between the adrenalectomy operation and the assay procedure, surgery was performed on the seventh and eighth days of the dietary regime in the second experiment.

Half the animals on each diet were injected with tryptophan several hours before sacrifice to test the adaptibility of TP. In the first experiment

animals were sacrificed on the eighteenth, twenty-first and twenty-third day of the dietary treatment. In the second experiment they were sacrificed on the twenty-first, twenty-second and twenty-third day. The data obtained in these experiments are presented in Table XVII. The rats fed diets lacking in thiamine showed a leveling off of their weight between the eighth and fourteenth days on the diet. After the fourteenth day all lost weight progressively, indicating the effect of the avitaminosis.

Table XVII

The Effect of Adrenalectomy and Thiamine on the Constitutive and Induced Levels of Tryptophan Pyrrolase

Diet fed rats in Experiment A is Diet 8: in Experiment B, Diet 7. Composition of the diets is given in section II Methods B ii. TP was assayed as described under section II Methods D: the reaction mixture of 4 ml. contained 2.25 mM L-tryptophan. The enzyme was contained in the supernatant fraction of a 12.5% rat liver homogenate in four determinations and in the supernatant fraction of a 7.6% rat liver homogenate in the remainder. TP activity is expressed as µMoles of kynurenine formed/hr./100 mg. dry weight of liver. The rats were injected intraperitoneally with 0.25 mMoles of L-tryptophan/100 g. body wt.in 2/3 isotonic saline to induce an increase in the TP activity. Control rats were injected with an equivalent volume of saline. In Experiment A the induction period was four hours: in Experiment B, three hours. Each figure in the table represents information from one animal.

Expt.	Operation	Thiamine	Inducer	TP activity	
A	Adrex[a]	-	-	1.84	1.24
				0.63	1.09
	Adrex	-	tryptophan	3.34	3.64
				5.09	1.55
	Adrex	+	-	2.15	0.26
				2.22	-
	Adrex	+	tryptophan	5.93	3.82
				2.32	1.09
	Sham	-	-		0.74
	Sham	-	tryptophan		5.61
	Sham	+	-		1.19
	Sham	+	tryptophan		3.19
B	Adrex	-	-	0.43	0.30
	Adrex	-	tryptophan	4.12	1.79
				1.45	-
	Adrex	+	-	0.53	0.22
				0.41	-
	Adrex	+	tryptophan	3.26	1.84
				2.12	-
	Sham	-	-	1.20	1.96
	Sham	-	tryptophan	3.66	4.15
	Sham	+	-	0.55	0.57
	Sham	+	tryptophan	4.78	2.82

[a] Adrex indicates adrenalectomy.

The expected decrease in the constitutive level of TP was not observed in the adrenalectomized rats of Experiment A (Table XVII) except for one rat receiving thiamine in its diet (0.26 μMoles kyn./hr./100 mg. dry liver). One other rat not receiving thiamine also had a decreased constitutive level (0.63 μM. kyn./hr./100 mg. dry liver) but the remainder were abnormally high when compared to the sham-operated control values. This finding suggests that some adrenal tissue remained in these rats as accessory cortical tissue or regeneration of the vestiges of incompletely removed adrenal glands. All the adrenalectomized rats except one on each diet showed marked increases in TP activity after tryptophan administration.

The values obtained in Experiment B present a markedly different picture. All the adrenalectomized rats had low constitutive levels of TP. One receiving thiamine had an activity comparable to the sham-operated controls. Adrenalectomy reduced the constitutive level of TP in the thiamine-deficient rats by at least a third. This effect of adrenalectomy supports the hypothesis that the increased constitutive levels observed occasionally (Table XVI) in thiamine-deficient rats is mediated hormonally. In all cases in Experiment B an increase in TP activity was found after the administration of tryptophan. These findings together with the results of the previous experiments confirm and extend the findings of others (183-186) that thiamine does not appear to be involved in the synthesis or activity of TP.

C. CONDITIONS CONCERNING THE INDUCTION OF TRYPTOPHAN PYRROLASE IN RAT LIVER SLICES.

Efimochkina (160) and Civen and Knox (161) have demonstrated increases in the activity of TP in rat liver slices. The former investigator was able to obtain nearly 5-fold increases in the TP activity of slices by incubating them for one hour in Krebs-Ringer-bicarbonate buffer containing 0.6% glucose and 6.13 mM L-tryptophan. Civen and Knox achieved 2- to 3-fold increases but have not published the details of their experiments.

The method for slicing rat liver and for incubation of the slices is given under section II Methods E. In the present experiments it was found that TP increases in activity during incubation at 37° in Krebs-Ringer-Phosphate buffer for a period of thirty minutes after which the rate of increase falls off or the activity remains constant for about one hour. This effect can be seen in Fig. 3. These data were compiled from four experiments in which the weight of groups of six slices (each group pooled for assay of the TP activity after incubation of the slices) varied from 460 to 766 mg. Each slice was incubated in 3 ml. of Krebs-Ringer-phosphate medium, pH 7.4, containing 0.5 mM DL-α-methyltryptophan which has been found to be equally as efficient as tryptophan for increasing the TP activity of slices. The activity of the slices after incubation was assayed according to the method described under section II Methods D and E. The data of Fig. 3 were obtained from experiments in which the gaseous phase was either 95% O_2 - 5% CO_2 or 100% N_2.

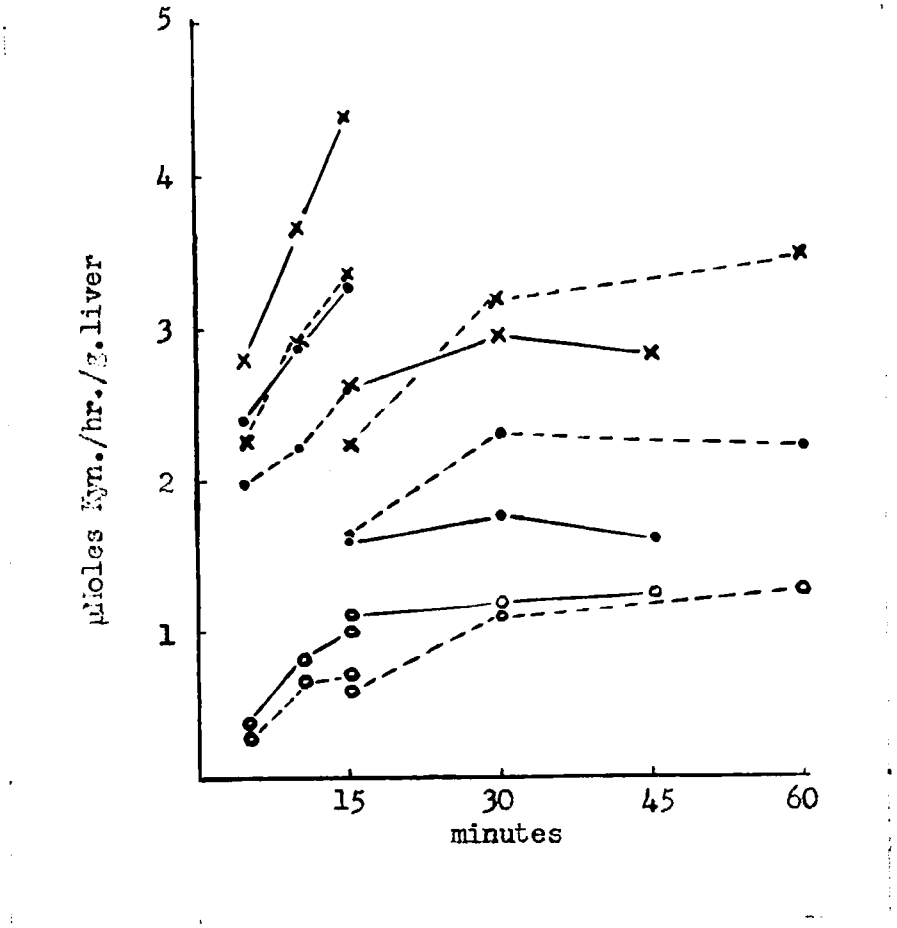

Figure 3: The effect of time on the tryptophan pyrrolase activity of rat liver slices incubated with and without DL-α-methyltryptophan (0.5 mM; total volume, 3 ml.) under 95% O_2-5% CO_2 and 100% N_2.

●—● is the activity of slices incubated under 95% O_2;
x—x is the activity of slices incubated with α-methyltryptophan under 95% O_2;
●--● is the activity of slices incubated under 100% N_2;
x--x is the activity of slices incubated with α-methyltryptophan under 100 N_2;
o—o is the difference in activity of slices incubated under 95% O_2 with and without α-methyltryptophan;
o--o is the difference in activity of slices incubated under 100% N_2 with and without α-methyltryptophan.

It can be seen from the graph that both the endogenous and induced (DL-α-methyltryptophan) levels of TP in the slices increase in activity up to about thirty minutes. The increment in activity due to the presence of inducer is most marked between five and fifteen minutes. Efimochkina (160) found a decrease in the activity of slices incubated without tryptophan for forty or sixty minutes. Fig. 3 shows that the endogenous activity actually increased in these experiments at least up to fifteen minutes after which the activity remained constant up to forty-five minutes under O_2 and sixty minutes under N_2. This increase in endogenous activity may be attributed to a release of tryptophan from the cells during the preparation of the slices. If such is the case the increase in the endogenous activity may represent adaptation. On the other hand endogenous TP may undergo a rapid turnover and for the first fifteen minutes of incubation, of the slices, TP being formed may be protected from degradation by the release of endogenous tryptophan. The activity of TP increases more rapidly in the presence of α-methyltryptophan (Fig. 3 - plot of increment). This suggests that this is the adaptation observed by Efimochkina (160) in slices.

Efimochkina's data (160) show that nearly linear increases in TP activity of slices can be induced by incubation of slices in a medium containing up to 6 mM L-tryptophan. In the present work this linearity was not observed. Fig. 4 is a scatter diagram of activities obtained from many experiments by incubating slices for one hour in the presence of various concentrations of tryptophan. Efimochkina's data were also converted to

the same standard and are plotted for comparison. These data were determined by measuring tryptophan disappearance rather than kynurenine production which may account for some of the discrepancies observed. The capacity of the tryptophan transaminative pathway may account for as much tryptophan degradation as the kynurenine-forming pathway (157).

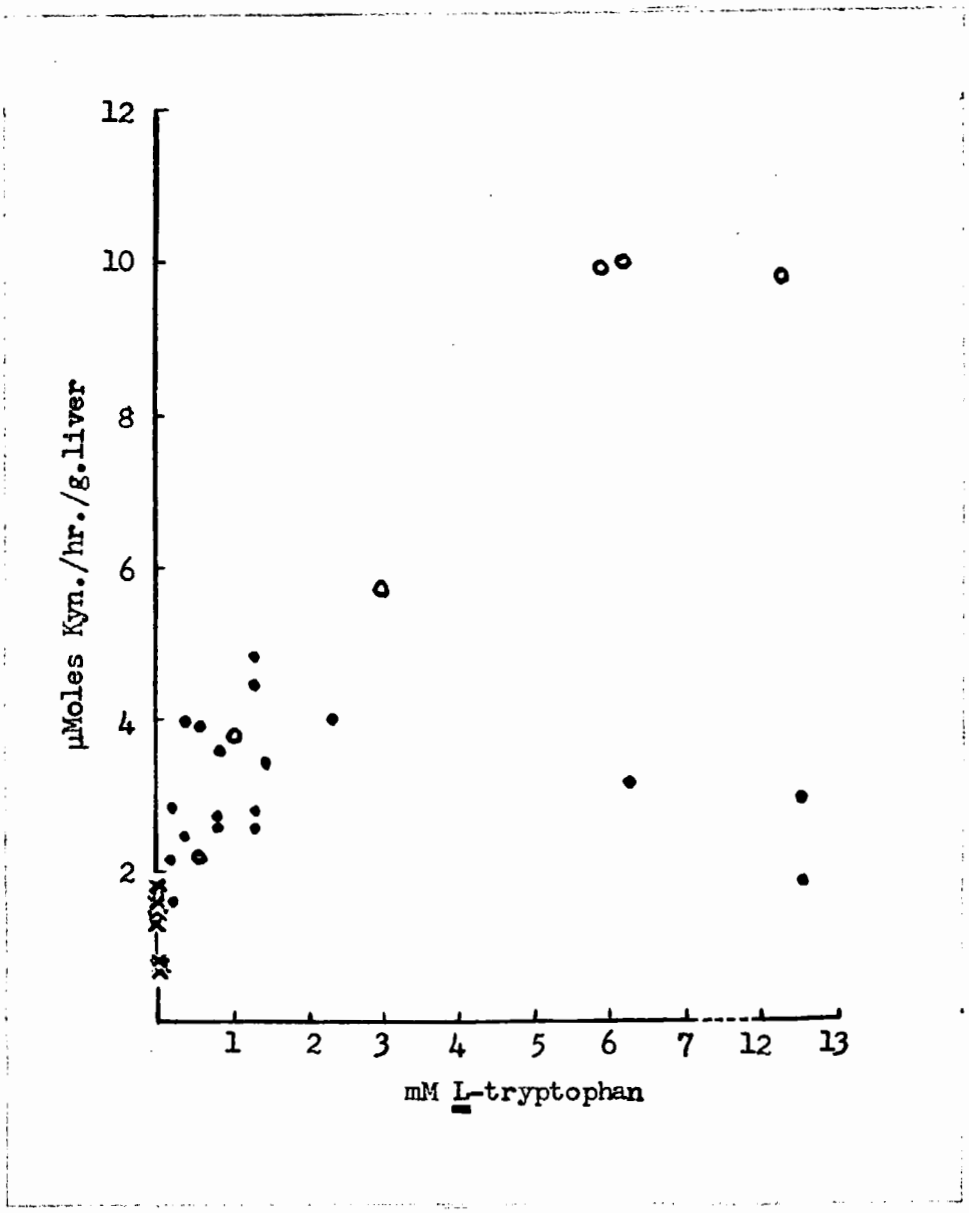

Figure 4: The effect of varying concentrations of L-tryptophan on the induction of tryptophan pyrrolase in slices: data were pooled from many experiments and for comparison, data from the experiments of Efimochkina (160) are included. Tryptophan pyrrolase activity is expressed as µMoles kynurenine formed/hour: the data of Efimochkina are expressed as µMoles tryptophan disappearing/hour.

- • activity of slices incubated with tryptophan;
- x activity of slices incubated without tryptophan;
- o activity of slices obtained in experiments of Efimochkina (160).

Figure 4 shows that increases in the activity of slices are obtained with concentrations of L-tryptophan in the incubation medium above 0.15 mM. These increases may be somewhat linear up to 1.25 mM L-tryptophan but do not appear, from the few values obtained using higher concentrations, to be linear above this concentration. Tryptophan may be utilized by the slices for three functions: (1) it may act in an inducer capacity, (2) it may serve as a substrate for degradative enzymes other than TP, and (3) as a substrate for TP. It would be expected that higher concentrations of tryptophan, if utilization is a controlling influence, would make more available for inducing purposes. However Lee has shown that the uptake of tryptophan from the plasma in intact rats keeps apace with its metabolism by the liver (157). This fact may explain the lack of linearity in adaptation at higher concentrations of tryptophan.

In some experiments considerable increases in the TP activity of slices incubated with tryptophan above the activity of control slices were obtained. The following table shows values obtained with slices weighing between 479 and 586 mg. (6 slices) which were incubated for 1 hour under 95% O_2 - 5% CO_2:

control	0.69 µMoles Kyn./hr./g. liver
0.75 mM L-tryptophan	2.71 µMoles Kyn./hr./g. liver
1.25 mM L-tryptophan	2.79 µMoles Kyn./hr./g. liver.

In many cases however, only small increases (less than 100%) were achieved. In order to reduce the possibility that these increases were due merely to protection of the enzyme by its substrate, incubation periods of ½ hour were

were adopted. During this period no decrease was found in the endogenous activity of TP in liver slices (Fig. 3). Low concentrations of inducer were also employed to assure linearity of the response.

Various isomers of tryptophan were investigated for their inducing ability in liver slices. Two experiments were performed; one in which the slices were incubated for ½ hour under 95% O_2 - 5% CO_2 and the second in which the slices were incubated under 100% N_2. The activities obtained under these conditions are listed in Table XVII.

Table XVII

The Activity of Tryptophan Pyrrolase in Slices After Incubation With Tryptophan and Tryptophan Analogues Under O_2 and N_2

The method of preparation and incubation of slices and assay of TP (reaction mixture 2) is described under section II Methods D and E. Incubation was carried out for ½ hr. The TP assay was carried out on a 14.3% homogenate of the slices; activity is expressed as μMoles kynurenine formed/hr./g. liver. In Experiment A, incubation of the slices was carried out under 95% O_2 -5% CO_2; in Experiment B, the atmosphere was 100% N_2.

Expt.	Addition*	Wt. of slices#	TP activity	Increment
A[a]	-	661	2.09	-
	0.3 mM D-tryptophan	667	2.56	0.47
	0.3 mM L-tryptophan	648	3.25	1.69
	0.3 mM DL-tryptophan	645	3.05	0.96
	0.3 mM DL- α-methyltryptophan	653	3.05	0.96
	0.3 mM DL-5-hydroxytryptophan	664	2.42	0.33
B	-	662	2.88	-
	0.3 mM D-tryptophan	632	2.84	0.04
	0.3 mM L-tryptophan	633	4.25	1.37
	0.3 mM DL-tryptophan	646	3.67	0.79
	0.3 mM DL- α-methyltryptophan	647	3.67	0.79
	0.3 mM DL-5-hydroxytryptophan	687	3.00	0.12

* isomers of tryptophan included in incubation medium of slices.

\# weight in mg. of each group of 6 slices pooled for TP assay.

In Experiment A of Table XVII an 80% increase in the activity of TP was obtained in the presence of L-tryptophan. In the presence of the D-isomer a 22% increase was obtained. In Experiment B where incubation of the slices was carried out under N_2 the D-isomer was entirely ineffective indicating that its activity probably occurs after inversion to the L-isomer under aerobic conditions. In both Experiments DL-tryptophan and DL- α-methyltryptophan were found to be

equally effective in promoting the activity of TP. DL-5-hydroxytryptophan also increases the activity of TP in slices to a small extent although it is an inhibitor of the enzyme (158).

The fact that the increase in TP activity can be achieved in slices incubated under N_2 as well as under O_2 (Fig. 3 and Table XVIII) leads to conjecture about the energy-requirement of this system. It would seem that substrate protection of a rapidly metabolized protein would explain equally well the differences in activity obtained in these experiments. Both α-methyltryptophan and 5-hydroxytryptophan which are inhibitors of TP activity in vitro (154,158) either protect the enzyme from degradation or stimulate an increased formation of the enzyme.

Four experiments were performed in attempts either to inhibit the increase in TP activity of slices incubated with tryptophan or to stimulate the increase due to incubation of the slices with tryptophan or α-methyltryptophan. These experiments and the results obtained are presented in Table XVIII.

Table XVIII

The Effect of Iodoacetate, Threonine, Serum and Casein on the Tryptophan Pyrrolase Activity of Liver Slices

Incubation of slices and assay of TP were carried out as described under section II Methods D and E. In Experiment A, incubation of the slices proceeded for 1 hr.; in B, C and D, for ½ hr. The gaseous phase was N_2 in A and B; 95% O_2 - 5% CO_2 in C and D. TP activity is expressed as μMoles kynurenine formed/hr./g. liver; the assay was carried out on a 12.5% rat liver slice homogenate in A; on a 14.3% homogenate in B, C and D. Reaction mixture 2 was used in TP assay.

Expt.	Addition*	Wt. of slices#	TP activity	Increment
A	-	732	3.10	-
	0.3 mM L-tryptophan	720	4.12	1.02
	1.0 mM iodoacetate	795	3.60	-
	0.3 mM L-tryptophan + 1.0 mM iodoacetate	748	4.61	1.01
(slices from rat fasted 24 hrs.)	-	580	5.51 (corr.[a]) 3.93	-
	0.3 mM L-tryptophan	567	6.70 (corr.[a]) 4.78	0.85
B	-	571	2.59	-
	0.3 mM L-tryptophan	606	3.90	1.31
	0.6 mM DL-threonine	577	2.31	-
	0.3 mM L-trypt. + 0.6 mM DL-threonine	610	3.82	1.51
C	-	678	1.73	-
	0.3 mM DL-α-methyltryptophan	688	2.52	0.79
	serum[b]	673	2.76	-
	serum[b] + 0.3 mM DL-α-methyltryptophan	663	3.23	0.47
D	-	526	1.96	-
	0.3 mM L-tryptophan	550	2.42	0.46
	casein[c]	509	1.41	-
	casein[c] + 0.3 mM L-tryptophan	568	2.32	0.91

* substances included in 3 ml. incubation medium

weight in mg. of each group of 6 slices pooled for TP assay

a value corrected for 40% increase in the number of cells per g. due to 24 hr. fast

b 3 ml. of incubation medium contained 2 ml. of serum

c 53 parts of NaCl constituent of Krebs-Ringer-Phosphate medium was replaced by 0.154 M casein hydrolysate supplemented with tyrosine, phenylalanine and cysteine.

In Experiment A of Table XVIII/which the slices were incubated under N_2 no inhibitory action could be attributed to the presence of iodoacetate in the incubation medium. Efimochkina (160) found an inhibition of the endogenous level of TP in slices in one experiment and a 30% decrease in the induced level of TP in a second experiment when 10^{-4}M 2,4-dinitrophenol was included in the incubation medium. A 100% increase of the endogenous activity was obtained without inhibitor in this investigator's experiments. The 50% increase in Experiment A due to the presence of tryptophan in the incubation medium did not demonstrate an energy requirement by this means.

In Experiment B of Table XVIII threonine was included in the incubation medium to see whether an effect similar to that observed by Chytil after the administration of threonine and tryptophan to intact animals (176) could be obtained. Chytil found that the administration of the 2 amino acids results in a higher TP activity than when tryptophan is administered alone. Only a very slight increase was found with slices and this was due mainly to a decrease in endogenous activity when threonine was included in the incubation medium.

It was thought that the addition of serum or casein hydrolysate to the incubation medium might stimulate the induction of TP in slices by providing amino acids or other possible biological activators (serum). Threonine dehydrase activity increases in isolated rat liver only when the perfusate contains a complete complement of amino acids (175). In Experiments C and D of Table XVIII it can be seen that no marked stimulation of TP adaptation in slices was obtained. The addition of serum to the incubation

medium increased both the endogenous and adapted levels of TP but the net effect was not one of increased adaptation. Casein decreased the endogenous TP activity of the slices obviating the interpretation of the increment in activity as due to an increased adaptation.

The interpretation of these data obtained from the incubation of slices with inducers and other agents as enzymatic adaptation cannot be considered conclusive. The adaptation can in all experiments be attributed to a protective action by the substrate or allied substance which is present in greater concentration in the cells of slices incubated in a medium to which these substances have been added. This view is supported by the fact that anaerobiosis and the presence of a glycolytic poison in the incubating medium did not affect the adaptation. The rate of increase in both endogenous and induced TP activities in the first fifteen minutes of incubation of the slices (Fig. 3) can be interpreted in two ways. Firstly in the case of the endogenous increase in activity, this enzyme which may have a rapid turnover may be protected from degradation by endogenous tryptophan released during the slicing procedure. In such a case while the protein is still being formed endogenously it is protected from breakdown during the first fifteen minutes, after which the degradative process keeps pace with its formation. Secondly, the rate of increase in TP activity of slices incubated with α-methyl-tryptophan exceeds that of the endogenous increase so that as well as protection there may well be an increased formation of TP.

D. EFFECT OF THIAMINE AND DIPHOSPHOTHIAMINE OF KYNURENINASE ACTIVITY IN VITRO

In the course of investigating the role of thiamine in the metabolism of tryptophan thiamine was tested for possible action in the kynureninase system in vitro. Dalgliesh (180,181) has found that pyridoxine deficient rats excrete metabolites which retain the side chain of tryptophan, kynurenine, hydroxykynurenine and xanthurenic acid. Anthranilic acid is not excreted. Furthermore in thiamine deficiency none of these metabolites is excreted and when both thiamine and pyridoxine deficiencies are imposed simultaneously, the metabolites characteristic of pyridoxine deficiency are greatly decreased. Dalgliesh concluded that thiamine deficiency imposes a block at the tryptophan pyrrolase level of tryptophan degradation. Junqueira and Schweigert (179) found that less of an administered tryptophan load was converted to nicotinic acid derivatives in animals deficient in thiamine than in normal animals. When a tryptophan load was imposed on rats fed diets from which thiamine was omitted an increase in the excretion of xanthurenic acid at the expense of kynurenic acid was noted by Porter, Clark and Silber (182). The excretion of kynurenine by these rats was not found to be significantly different from the excretion by control rats. In view of the results obtained in the investigation of the effect of thiamine on TP activity it was decided to investigate the effect of this vitamin on kynureninase.

i) Observation on the Inhibitory Action of Thiamine and Diphosphothiamine

Experiments were carried out to determine the effect of thiamine and diphosphothiamine on the kynureninase system in vitro. The enzyme was

contained in the supernatant fraction of fresh rat liver extracts. Kynureninase *in vivo* is partially resolved with respect to its coenzyme (189) and for this reason all enzyme preparations were pre-incubated with pyridoxal phosphate to assure saturation before the addition of substrate or inhibitor substances. The procedure employed for determining kynureninase activity is given under section II Methods F.

Both thiamine and diphosphothiamine (DPT) have been found to inhibit the formation of anthranilic acid from kynurenine in the enzyme system *in vitro*. The results of four experiments are summarized in Table XIX.

Table XIX

Effect of Thiamine and Diphosphothiamine on the Formation of Anthranilic Acid by Rat Liver Extracts in vitro

Assay system is described under section II Methods F: 120 µg. and 12 µg. of pyridoxal phosphate were pre-incubated with the liver fraction in Experiments 1-3 and Experiment 4 respectively. DL-Kynurenine was substrate. Thiamine (B_1) and diphosphothiamine (DPT) and ~~D~~-kynurenine were added to the reaction mixture after pre-incubation of the enzyme preparation with pyridoxal phosphate. Formation of anthranilic acid is expressed as µMoles.

Expt.	Incubation period (mins.)	Initial concentration µMoles Kyn./ml.	Concentration inhibitor µMoles/ml.	Formation Anth. Acid Inhibitor -	Inhibitor +	Percent inhibition
1	30	0.75	-	0.04	-	-
	60	0.75	-	0.11	-	-
	30	3.00	2.5 (DPT)	0.13	0.07	46
	60	3.00	2.5 (DPT)	0.26	0.15	41
2	30	1.50	2.5 (DPT)	0.10	0.04	56
	30	1.50	1.25(B_1)		0.07	24
	30	1.50	12.5 (B_1)		0.02	80
	60	1.50	2.5 (DPT)	0.19	0.08	59
	60	1.50	1.25(B_1)		0.14	30
	60	1.50	12.5 (B_1)		0.04	79
3	30	1.50	2.5 (DPT)	0.21	0.08	60
	60	1.50	2.5 (DPT)	0.38	0.16	59
	90	1.50	2.5 (DPT)	0.54	0.21	62
4	65	0.75	2.5 (DPT)	0.22	0.08	64
	95	0.75	2.5 (DPT)	0.32	0.12	63
	65*	0.75	2.5 (DPT)	0.23	0.09	63
	95*	0.75	2.5 (DPT)	0.31	0.09	62

* liver extracts were dialysed for two hours against 0.01 M K_2HPO_4-KH_2PO_4, pH 7.4

Anthranilic acid formation from kynurenine is essentially linear under the conditions used in these experiments for incubation periods up to 90 minutes as can be seen from the results of Experiments 3 and 4, Table XIX. The substrate employed in these experiments was DL-kynurenine but only the L-isomer is utilized by kynurenine (189). In the presence of 2.5×10^{-3}M DPT, inhibition of anthranilic acid formation ranged from 41 to 64%. In Experiments 2 and 3 120 µg. of PLP (pyridoxal phosphate) were used to saturate the enzyme with its coenzyme while in Experiment 4 only 12 µg. were used. Comparing the anthranilic acid formation in the presence of DPT at 60 minutes in Experiments 2 and 3 with the formation at 65 minutes in Experiment 4 it can be seen that the same degree of inhibition was obtained (Expt. 2 - 59%, Expt. 3 - 60%, Expt. 4 - 64%). Inhibition was not relieved by ten times the concentration of pyridoxal phosphate in these experiments. Thiamine at concentrations of 1.25 and 12.5×10^{-3} M inhibited kynureninase by 24-30% and 79-80% respectively.

ii) Characterization of the Inhibition of Kynureninase by Diphosphothiamine

The investigation of the inhibitory action of DPT on kynureninase was extended in an attempt to characterize the nature of this effect. Several factors became apparent: the inhibition is dependent on the concentration of DPT and secondly, the inhibition is relieved by increasing the substrate concentration. These conditions are exemplified in the experiments summarized in Table XX.

Table XX

Effect of Substrate and Inhibitor Concentration on the Inhibition of Kynureninase by Diphosphothiamine

Assay system is described under section II Methods F: 20 µg. of pyridoxal phosphate were pre-incubated with the liver extract for 30 minutes in Experiment 1, and 20 minutes in Experiment 2. DL-kynurenine was substrate in Experiment 1, L-kynurenine in Experiment 2. Both substrate and diphosphothiamine (DPT) were added to the reaction mixture after pre-incubation with pyridoxal phosphate. Formation of anthranilic acid is expressed as µMoles/60 minutes: this value is corrected for endogenous control values (without substrate).

Expt.	Initial concentration µMoles Kyn./ml.	Concentration DPT µMoles/ml.	Formation Anthranilic Acid inhibitor -	inhibitor +	Percent inhibition
1	0.75	2.5	0.24	0.07	69
	0.75	5.0		0.03	88
	0.75	10.0		0.00	100
	1.50	2.5	0.35	0.12	65
	1.50	5.0		0.05	86
	1.50	10.0		0.01	99
2	0.375	1.0	0.24	0.15	38
	0.375	2.5		0.09	60
	0.375	5.0		0.04	83
	0.75	1.0	0.48	0.31	37
	0.75	2.5		0.19	61
	0.75	5.0		0.08	84
	1.875	1.0	0.71	0.59	17
	1.875	2.5		0.36	49
	1.875	5.0		0.15	79
	3.75	1.0	0.77	0.81	(+) 5
	3.75	2.5		0.55	29
	3.75	5.0		0.33	42

The effect of increasing the inhibitor (DPT) concentration on the activity of kynureninase is exemplified in Experiment 1 and 2 of Table XX. In Experiment 1, where 0.75 and 1.5 x 10^{-3} M DL-kynurenine was employed as substrate, 2.5 x 10^{-3} M DPT inhibited the formation of anthranilic acid to the extent of 65 to 69%. A concentration of 10^{-2} M affected a complete inhibition of the enzyme. In Experiment 2 the same effect can be observed with equivalent concentrations of the active isomer (L-kynurenine). Relief of the inhibition with concentrations of L-kynurenine above 0.75 x 10^{-3} M is manifested in this experiment. The 37% inhibition of anthranilic acid formation found using 0.75 x 10^{-3}M L-kynurenine and 1 x 10^{-3}M DPT was abolished when the substrate concentration was increased to 3.75 x 10^{-3}M. At lower concentrations of substrate, 0.375 x 10^{-3}M L-kynurenine (0.75 x 10^{-3}M DL-kynurenine) and 0.75 x 10^{-3} M L-kynurenine (0.5 x 10^{-3}M DL-kynurenine) the relief of inhibition cannot be observed.

These and other data have been plotted in various ways to emphasize the effect of varying the substrate and DPT concentrations on the formation of anthranilic acid. Fig. 5 shows the effect of increasing concentrations of DPT on the inhibition of kynureninase at different substrate concentrations.

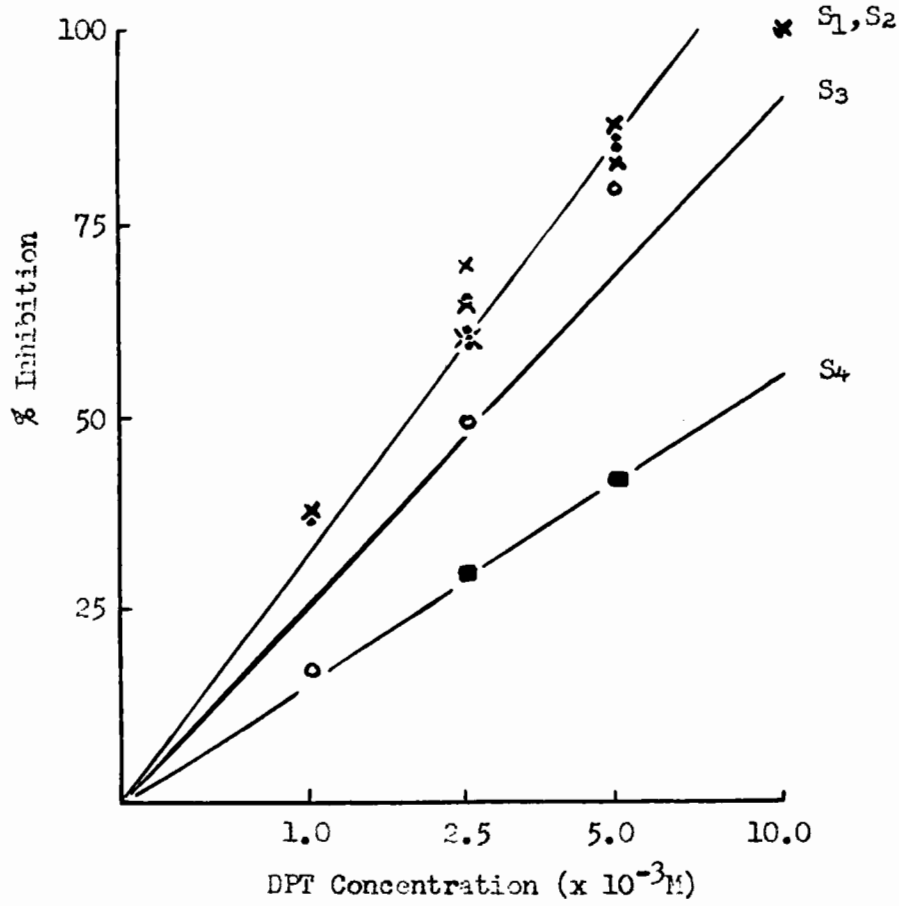

Figure 5: The effect of increasing the concentration of diphosphothiamine on the inhibition of kynureninase assayed at various substrate concentrations. The ordinate represents the percent inhibition of anthranilic acid in the presence of DPT. The abscissa represents the concentration of DPT present in the reaction time.

x 0.375 mM L-kynurenine substrate concentration (S_1);
• 0.75 mM L-kynurenine substrate concentration (S_2);
o 1.875 mM L-kynurenine substrate concentration (S_3);
□ 3.75 mM L-kynurenine substrate concentration (S_4).

A similar relationship exists between the concentration of substrate and inhibitor when thiamine is employed an inhibitor as when DPT is employed. This relationship is demonstrated in the data summarized in Table XXI.

Table XXI

Effect of Substrate and Inhibitor Concentration on the Inhibition of Kynureninase by Thiamine

Assay system is described under section II Methods F: 10 µg. of pyridoxal phosphate were pre-incubated with the liver extract for 20 minutes. The substrate was L-kynurenine. Both substrate and thiamine were added to the reaction mixture after pre-incubation with pyridoxal phosphate. Formation of anthranilic acid is expressed as µMoles/60 minutes: this value is corrected for endogenous control values.

Initial concentration µMoles Kyn./ml.	Concentration Thiamine µMoles/ml.	Formation Anthranilic Acid Thiamine -	Thiamine +	Percent Inhibition
0.375	2	0.18	0.14	23
0.375	5		0.08	54
0.375	10		0.01	95
2.25	2	0.53	0.46	13
2.25	5		0.42	22
2.25	10		0.26	52

iii) Kinetics of Kynureninase

The previous data have shown that the inhibitory effect of DPT and thiamine on kynureninase is relieved by higher concentrations of substrate (kynurenine). This effect would denote competitive inhibition: The data of Experiment 2, Table XX were plotted according to the method of Lineweaver and Burk (211). This plot can be seen in Fig. 6.

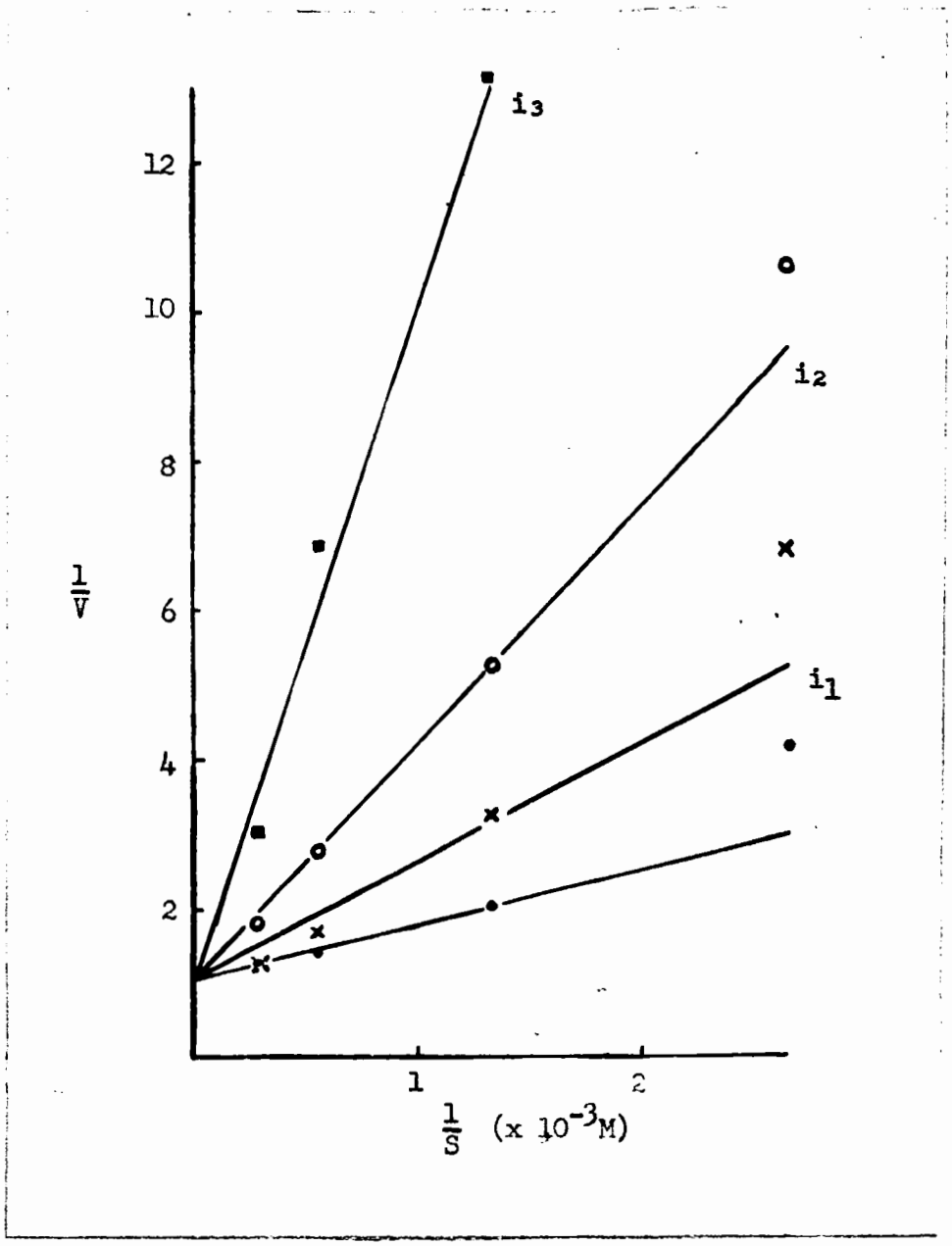

Figure 6: Plot characterizing inhibition of kynureninase by diphosphothiamine according to the method of Lineweaver and Burk (211). The effect of kynurenine concentration (S) on the rate of the kynureninase reaction (V): the effect of varying concentrations of DPT (i) on the rate of the kynureninase reaction.

v = μMoles anthranilic acid formed/60 min.;
s = initial concentration of L-kynurenine;
x = 1.0×10^{-3}M DPT = i_1
o = 2.5×10^{-3}M DPT = i_2
□ = 5.0×10^{-3}M DPT = i_3

The effect of kynurenine concentration and DPT on kynureninase activity plotted in Fig. 6 reveals two facts: (1) at concentrations of substrate above 0.375×10^{-3} M all the slopes share a common intercept and (2) in the presence of increasing concentrations of inhibitor the slopes increase. These findings suggest that DPT inhibition of kynureninase is formally competitive when the substrate concentration is 0.75×10^{-3} M or higher. The competition may involve the apo-enzyme or the kynureninase-pyridoxal phosphate complex. Because no valid conclusion concerning the nature of the inhibition between substrate and inhibitor can be drawn when a cofactor is involved unless the effect of the inhibitor is investigated as a function of both substrate and cofactor concentration (212), the competitive nature of the DPT inhibition can only be considered as provisional.

With this reservation in mind, the K_s (substrate concentration at half maximum velocity) was determined from the data of Experiment 2, Table XX according to the method of Lineweaver and Burk (211):

$$\text{Slope} = \frac{K_s}{V_{max.}}$$

$$V_{max.} = 0.912$$

$$\text{Slope} = 0.734$$

$$K_s = 0.67 \times 10^{-3} \text{ M.}$$

This value is somewhat higher than the value found by Knox (0.4×10^{-3} M), using L-kynurenine (189). In experiments where no relief of DPT inhibition could be ascertained by doubling the substrate concentration (Table XX, Experiment 1 and the two lowest substrate concentrations of Experiment 2)

it is plausible that suboptimal substrate concentrations may account for this effect. The dissociation constant (K_s) is approximately 0.67×10^{-3} M a value nearly double the lowest concentrations of L-kynurenine used in these determinations.

These data suggest a possible interpretation for Dalgliesh's observations on the fate of tryptophan administered to rats suffering from thiamine or thiamine and pyridoxine deficiencies (180,181). In normal rats Dalgliesh found that after the administration of tryptophan xanthurenic acid and traces of kynurenine and anthranilic acid are excreted in the urine. In thiamine deficient rats none of these metabolites are excreted, and doubly deficient rats excrete the metabolites characteristic of pyridoxine deficiency only in trace amounts. The excessive excretion of xanthurenic acid in pyridoxine-deficient rats was found to be reduced to control levels when the animals were doubly deficient. Dalgliesh attributed the effect of thiamine deficiency to a block at the level of tryptophan pyrrolase because neither formylkynurenine nor any of the metabolites following it are excreted in thiamine-deficient rats following a load of tryptophan. Tryptophan pyrrolase activity has been shown to be unaffected by thiamine deficiency and in view of the findings on the effect of this vitamin and DPT on kynureninase an alternative interpretation is suggested: thiamine and DPT may exert an endogenous control of kynureninase activity. In the thiamine-deficient rats of Dalgliesh (181) the controlling influence of these substances would be reduced thus making it possible for larger amounts of tryptophan to be efficiently metabolized to products below the level of kynureninase.

Kynureninase is readily resolved with respect to its coenzyme and this occurs in pyridoxine deficiency. However in this deficiency two factors may be affecting kynureninase: (1) deficiency of its coenzyme and (2) inhibition of kynureninase by the additional inhibitory influence of thiamine or DPT present in the organ. These two factors could account for the excessive excretion of xanthurenic acid due to a shunt of a large proportion of the substrates of kynureninase through the transaminative pathway. In a double deficiency this inhibitory influence of thiamine and DPT is reduced and the effect of the pyridoxine deficiency on kynureninase is not as marked. Traces of kynurenine, hydroxykynurenine and xanthurenic acid (no more than in the control rats) are excreted as a result. This interpretation is subject to several reservations. The effect of thiamine and DPT on kynureninase has not been investigated with 3-hydroxykynurenine as substrate. A very important consideration too is whether conditions in the cell are conducive to a controlling influence by these substances on kynureninase activity. These conditions include availability at the site of kynureninase action of thiamine and DPT in sufficient concentration to affect an inhibitory action.

IV DISCUSSION

A. THE INFLUENCE OF DIETARY TRYPTOPHAN ON THE 5-HYDROXYINDOLES OF THE RAT

The endogenous 5-hydroxyindoles of young rats have been found to be very sensitive to the omission of tryptophan from the diet. After one day on such a diet the 5-HT stores of the small intestine suffered a decrease of almost 50% (Table VI_B). The effect of tryptophan deprivation was also revealed in the urinary excretion of 5-HIAA which is considered to reflect the endogenous turnover of 5-hydroxyindoles (134,135). A similar decrease in the urinary 5-HIAA excretion of rats fed diets deficient in tryptophan also occurred after one day (Table VII).

Following the first 24 hours of the dietary regime in which tryptophan was removed from the diet the rats appeared to draw on an endogenous tryptophan supply which enabled them to maintain their stores of 5-HT in the small intestine for at least seven to fourteen days (Table VI_B). The difference between the levels of 5-HT in the deficient and control rats was maintained throughout this period. The urinary excretion of 5-HIAA by rats fed diets from which tryptophan was omitted reflected the changes found in the small intestine. This similarity was demonstrated on the first, fourth and fifth days of the dietary regime (Table VII). The 5-hydroxyindoles of humans and dogs do not appear to be as sensitive to the level of dietary tryptophan as those of rats since alterations in this level fail to alter appreciably the urinary excretion of 5-HIAA (107[b]).

The hydroxylating system forming 5-hydroxyindoles operates maximally on endogenous stores of tryptophan (107[b]) and in humans 3% of the dietary

tryptophan is estimated to be metabolized as 5-HT (28). Assuming that the urinary 5-HIAA excretion of rats on diets which contained a known amount of tryptophan is a reflection of the tryptophan content of the diet, the dietary tryptophan metabolized as 5-hydroxyindoles can be estimated from the data in Table VII. The data are taken from the fourth and fifth days when the dietary tryptophan made up 1% and 0.4% of the diets respectively. The excretion of 5-HIAA on each occasion represented the pooled excretion of four rats corrected to the standard, 5-HIAA/100g./24 hrs. Assuming that a 100 gram rat consumes approximately 10 grams of diet daily then the dietary tryptophan content metabolized as 5-hydroxyindoles is as follows:

diet contains 0.4% tryptophan = 40 mg. tryptophan/day = 0.1% as 5-hydroxy-indoles,

diet contains 1.0% tryptophan = 100 mg. tryptophan/day = 0.07% as 5-hydroxy-indoles.

This calculation takes into account that only about one third of 5-HT metabolized is excreted as urinary 5-HIAA (134,107[b]). Although it appears from this calculation that almost the same percentage of dietary tryptophan is utilized by the rats for 5-hydroxyindoles when the diet consists of 1% tryptophan as when it consists of 0.4% tryptophan this cannot be considered conclusive. These determinations of 5-HIAA excretion were carried out in separate dietary experiments in which the effect of tryptophan deprivation was investigated. Although in both experiments a similar relationship between 5-HIAA excretion and the tryptophan content of the diet was found the rats deprived of tryptophan excreted markedly different amounts of 5-HIAA in the two experiments. For this

reason the common basis for comparing the effect of the dietary level of tryptophan in the two experiments, 5-HIAA excretion by tryptophan deprived rats, was not reliable.

The effects of tryptophan deficiency on the 5-HT content of the small intestine of rats has also been investigated by Eber and Lembeck (105). Several discrepancies are apparent in the results of their experiments as compared to the ones reported here. The first discrepancy is that of two dietary studies conducted by these investigators in one a decrease of over 50% in the intestinal 5-HT was detected on the fifteenth day but in a second only after 31 days of tryptophan deprivation. The second discrepancy lies in the 5-HT content of the small intestine of rats receiving tryptophan in their diets. Eber and Lembeck found values of 1.79 ± 0.51 and 2.96 ± 0.65 µg. 5-HT/gram of small intestine. The 5-HT content of the small intestine of seven rats was determined in the course of the present investigation (Table VI_B) and found to vary between 3.55 and 7.16 µg. 5-HT/g. The concentration of 5-HT found by Eber and Lembeck in the small intestine of rats approximates the values found for the whole intestine in the dietary experiments summarized in Table VI_A) and in control experiments when a concentration of 2.39 ± 0.43 µg. 5-HT/gram of whole intestine was found. A difference in the strain of rats used may account for some of the discrepancies apparent in comparing the results of these investigators with those of this thesis. A second factor which could conceivably influence the control values of 5-HT is the level of tryptophan contained in the diet of the rats.

Reserpine was employed in two experiments in an attempt to augment the effects of a dietary tryptophan deficiency on the urinary 5-HIAA excretion of rats. The administration of 1 to 2 mg. reserpine/kg. to rabbits effects a 60 to 75% decrease in the 5-HT stores of the small intestine (138) and effects the release of 5-HT from the brain (139) and platelets (140). An increased excretion of 5-HIAA occurs as a result of the liberation and metabolism of the endogenous stores of 5-HT. Following the administration of 3 mg. reserpine/kg. to dogs the urinary 5-HIAA returns to normal levels after approximately eight hours (205). A similar effect is elicited in rats following the administration of reserpine (144). No effect of the urinary 5-HIAA excretion could be detected one or two days following the administration of 1 or 2 mg. reserpine/kg. (Tables VIII and IX). In these experiments reserpine was administered to the rats on two occasions to effect a depletion of their body stores of 5-HT. Rats receiving diets from which tryptophan was omitted, showed no augmentation of the deficiency as a result of the reserpine treatment. These findings confirm those of Erspamer and Ciceri (144) and Brodie et al. (145) who showed that reserpine does not interfere in the biosynthesis of 5-HT. In spite of the deprivation of tryptophan and the additional effect of reserpine administration, the endogenous tryptophan stores appeared to be capable of sustaining 5-hydroxyindole synthesis for periods of at least seventeen to twenty-one days as evidenced by the continued excretion of considerable amounts of 5-HIAA (Tables VIII and IX).

The data on the effect of tryptophan deficiency on 5-hydroxyindole levels in the intestine and urine of rats can also be used to estimate the

daily metabolism of these substances. A mean value of 2.39 µg. 5-HT/g. of whole intestine was obtained in control experiments; in a 100 gram rat whose intestine weighs about 6 grams, the 5-HT content would be approximately 14 ug. In one experiment the mean excretion of 5-HIAA by four rats on a complete diet was found to be 10.8 µg./100 g./24 hrs.; when corrected for slight differences in molecular weight and the percentage of 5-HT metabolized as 5-HIAA (134), the 5-HIAA excretion represented the metabolism of 29.8 µg. 5-HT/100 g./24 hours. On the basis of this estimation an amount of 5-HT corresponding to the content of the whole intestine could be metabolized every 12 hours. Erspamer has estimated that an amount of 5-HT equivalent to the content of the entire gastro-intestinal tract is metabolized every 8 to 9 hours (Table II, 134). A 50% decrease in the 5-HT content of the small intestine and the urinary 5-HIAA excretion was demonstrated in experiments in which rats were deprived of tryptophan for 24 hours (Tables VI_B and VII). Taking into account that tryptophan from both exogenous and endogenous sources influences the 5-HT levels of the rat in the first 24 hours after the removal of tryptophan from the diet probably more than 50% of the 5-HT of the small intestine is replaced every 24 hours. Udenfriend and Weissbach have determined the half life of intestinal 5-HT in the rabbit as 17 hours (Table I).

B. UTILIZATION OF D-TRYPTOPHAN BY THE RAT

The early investigations of Berg and his associates (12,14) and du Vigneaud *et al.* (13) established that D-tryptophan is as efficient as the L-isomer in supporting the growth of young rats. The ability of D-tryptophan

to replace the L-isomer has been investigated by several different experiments. When the dietary tryptophan was supplied in the D-isomeric form, the urinary 5-HIAA excretion was found to be as well maintained as with the 'natural' L-tryptophan. The activity of TP and the changes in the liver and body weight of these animals were determined: the results were subjected to statistical analysis. The D-isomer was found to be as efficient as the L-isomer for the maintenance of growth, liver weight and TP activity.

The results of experiments performed *in vitro* suggest that the activity of D-tryptophan is dependent on its inversion to the L-isomer. The activity of TP in liver slices was determined after incubation with D-tryptophan under O_2 and N_2 (Table XVII). When the incubation was carried out under O_2 a 25% increase in the activity of TP was observed over the control value obtained by incubating slices without tryptophan. When the slices were incubated under N_2 no such stimulation of TP activity was obtained with D-tryptophan. D-tryptophan is not a substrate for this enzyme (39) but when administered to rats, it is incorporated as L-tryptophan into the tissue proteins (31). The inversion of D-tryptophan to L-tryptophan probably occurs through oxidative deamination by D-amino acid oxidase, an enzyme found in liver and kidney (34), to indole-3-pyruvic acid which can then be converted to L-tryptophan through a transamination reaction. The tryptophan-α-ketoglutarate transamination reaction has recently been studied more extensively by Lin, Civen and Knox (35,36). The fact that indolepyruvic acid is an effective replacement for tryptophan in the diet of rats (18) is additional evidence for the inversion of D- to L-tryptophan by this route.

C. THE INFLUENCE OF THIAMINE ON TRYPTOPHAN METABOLISM

Thiamine has been known for some time to be involved in the metabolism of tryptophan but the nature of its role has not been elucidated. Junqueira and Schweigert have shown that in thiamine deficiency there is a decreased conversion of tryptophan to nicotinic acid derivatives in the rat (179). Dalgliesh has found that the administration of a tryptophan load to thiamine-deficient rats does not give rise to a normal excretory pattern of tryptophan metabolites; normal animals excrete traces of kynurenine, anthranilic acid and xanthurenic acid, thiamine-deficient rats do not (180,181). In pyridoxine deficiency a block is imposed at the kynureninase level of tryptophan degradation and only the metabolites containing the side chain of tryptophan are excreted. Dalgliesh has found that when rats are deprived of both vitamins the urinary metabolites characteristic of pyridoxine deficiency are greatly decreased. On this basis Dalgliesh concluded that thiamine is involved at the TP level of tryptophan degradation.

The influence of thiamine on TP has been investigated by several means in the course of this present research. In an attempt to detect a direct action of the vitamin on TP it was injected intraperitoneally into rats, alone and with tryptophan; it was found neither to elicit an increased activity of the liver TP nor to stimulate the response due to the administration of tryptophan. TP is very labile in liver homogenates although it is stabilized to a considerable extent in the presence of its substrate (39,155). In this respect the coenzyme form of thiamine, diphosphothiamine, was investigated

for a possible stabilizing or re-activating action on this enzyme. It was found to be without influence on the oxidation of tryptophan *in vitro* and furthermore to have no re-activating effect on aged or dialysed liver preparations. The results of these experiments apparently exclude the possibility of a direct influence of thiamine or DPT on the activity of TP *in vitro*.

The possibility that thiamine may have some direct action on the biosynthesis of TP has been investigated in dietary deficiency studies by several groups of investigations. Ginoulhiac (183) could find no evidence for a direct effect of thiamine on the constitutive level of the enzyme in thiamine-deficient rats even when the deficiency progressed to the stage where the animals were suffering from polyneuritis. In similar experiments the adaptive formation of TP, whether evoked by the administration of tryptophan (184) or hormonally (186), has been shown to be unaffected by deprivation of tryptophan. These findings have been confirmed and extended. In multifactorial dietary experiments the constitutive level of TP in the livers of rats deprived of thiamine and of both thiamine and tryptophan has been compared to the level of TP obtained from animals which received a fully supplemented diet. Thiamine was found to be without direct effect on the liver TP activity. Furthermore, no interaction of thiamine and tryptophan was evinced although the influence of tryptophan deprivation on this enzyme was revealed. Because of the stressing effect of thiamine deprivation and the possibility that a hormonally evoked stimulation of TP formation might conceal the direct influence of thiamine on this enzyme, dietary experiments were carried out with intact and adrenalectomized rats. Thiamine deficiency was

found to evoke a hormonal stimulation because the elevated levels of TP activity found in intact rats deprived of thiamine were not found in adrenalectomized rats similarly deprived of thiamine. On the other hand this effect of the deficiency was not evident in the adaptive formation of the enzyme following the administration of tryptophan. Again, a requirement for thiamine in the biosynthesis of TP could not be demonstrated (Tables XVI and XVII).

In order to point up any influence of thiamine on TP activity, the thiamine antimetabolites, oxythiamine and pyrithiamine, were administered to rats daily for twelve days. During this time symptoms of the treatment were evidenced by changes in body weight and by developing polyneuritis in the pyrithiamine-treated rats. These animals showed elevated constitutive levels of TP, an effect probably elicited through the adrenal cortex due to the stress of the treatment. In addition the response of the pyrithiamine-treated rat to the administration of tryptophan was considerably enhanced as compared to the response of the control rat. This effect could be attributed to the two known mechanisms responsible for the adaptation: (1) the direct response due to the administration of tryptophan and (2) an additional hormonal stimulation of the enzyme due to the stress of administering tryptophan to an already severely stressed animal. The rats treated with oxythiamine appeared to be much healthier animals and the adaptive response to tryptophan administration of one of these rats was less than half that of the rat treated with pyrithiamine. On the other hand the oxythiamine-treated rat responded to the administration of tryptophan by an increased TP activity only about 70% of that of the control rat. Nevertheless these antimetabolites did not appear

to interfere with the liver TP activity sufficiently to warrant their further investigation.

Because no convincing data were obtained to suggest that thiamine is involved at the tryptophan pyrrolase level of tryptophan degradation, it was considered worthwhile to investigate the influence of thiamine below this level. Furuta has found that after the administration of oxythiamine to rats for 27 days the activities of the systems forming and degrading kynurenine are reduced to the extent of about 40 to 70% (207). Furthermore, he found that the substrate of the transamination reaction, hydroxykynurenine, and the ultimate product of this reaction, xanthurenic acid, are excreted by rats treated in this way. This finding suggests that the administration of the antimetabolite to rats imposes a block at the level of tryptophan degradation where the side chain of tryptophan is cleaved, i.e. at the level of kynureninase action.

Experiments <u>in vitro</u> have shown that thiamine and DPT do influence the activity of kynureninase. Concentrations of thiamine as low as 1.25×10^{-3}M and of DPT at least as low as 1×10^{-3}M inhibited the formation of anthranilic acid by liver extracts considerably (Table XIX and XX). This inhibition was relieved by increasing the substrate concentration (Fig. 5) but not by increasing the concentration of pyridoxal phosphate, the coenzyme of kynureninase (Table XIX). The nature of the inhibition of kynureninase by DPT has been provisionally classed as competitive (Fig. 6) but whether the inhibition involves the apoenzyme or the kynureninase-pyridoxal phosphate complex has not been elucidated. If the coenzyme is involved the conditions

necessary to achieve inhibition may be considerably altered depending on the degree of resolution of the enzyme.

In view of the inhibitory effect of DPT and thiamine on kynureninase in vitro it is conceivable that a similar influence may affect the activity of kynureninase in vivo. The concentration of thiamine in beef liver is of the order of 450 µg./100 grams (214). Assuming that the rat liver concentration is similar to that of beef, the thiamine level of rat liver can be estimated as approximately 2×10^{-5}M. This concentration is much lower than that found to inhibit the enzyme in vitro. However, certain discrepancies between the activity of the enzyme in vitro and in vivo may affect the conditions necessary for this inhibition. Kynureninase in vivo is considered to be saturated with respect to its coenzyme to the extent of about 75% (189). The inhibitors in the in vitro study were not pre-incubated with the partially resolved enzyme but always added to the assay system after saturation of kynureninase with pyridoxal phosphate. For this reason if the coenzyme is involved in some way in the inhibitory influence of DPT the conditions necessary to achieve inhibition of the partially resolved enzyme may be considerably altered.

In the light of these findings an alternative interpretation is offered to explain the effects of thiamine and pyridoxine deficiency observed by Dalgliesh (180,181). This investigator employed the technique of paper chromotography to determine certain of the metabolites of tryptophan which appear in the urine following the administration of a tryptophan load to rats. Two factors are known which influence the metabolism of a tryptophan load: (1) the adaptive increase in TP activity thereby facilitating degradation of

tryptophan via the route forming kynurenine and (2) an adaptive increase of tryptophan-α-ketoglutarate transaminase which facilitates the degradation of tryptophan via indolepyruvic acid.

In addition to the adaptation mechanisms the degree to which the enzymes degrading tryptophan are saturated with their coenzymes is affected by the availability of their precursor vitamins. In this respect the utilization of α-ketoglutarate by the tissue of thiamine-deprived rats is increased by the addition of thiamine (215). Thiamine on conversion to diphosphothiamine serves as coenzyme for the decarboxylation of this acid (216). Evidence for a decreased degradation of α-ketoglutarate in thiamine deficiency is the accumulation of glutamic acid in the liver and kidney: aspartic acid in these organs is decreased (127). Glutamic acid arises from (a) transamination between alanine or aspartic acid and α-ketoglutarate and (b) the amination of α-ketoglutarate. The well- known increase in pyruvic acid and the possible accumulation of α-ketoglutarate in the tissues of thiamine-deprived rats would tend to favour transamination mechanisms. Pyridoxine deficiency in rats is characterized by the excretion of the metabolites of tryptophan having the original side chain intact. These include kynurenine, hydroxykynurenine and the rearranged products of their transamination, kynurenic and xanthurenic acids. Pyridoxal phosphate is the coenzyme for both the transaminase and kynureninase enzymes, but in depriving rats of the precursor of this coenzyme, pyridoxine, inhibition of the latter enzyme is first observed (180,181).

In order to interpret Dalgliesh's findings concerning the role of

thiamine in tryptophan metabolism a multiple effect must be visualized:

(1) both TP and the tryptophan transaminase enzymes are increased in activity by the administration of a tryptophan load;

(2) in thiamine deficiency however more tryptophan is probably channeled through the tryptophan transaminase pathway due to the accumulation of one of the reactants, α-ketoglutarate;

(3) kynureninase activity may be more efficient owing to the relief of the inhibitory influence of DPT and thiamine by the deficiency.

The fact that there is less conversion of tryptophan to nicotinic acid derivatives in thiamine-deficient rats (179) lends support to the influence of factor 2 (above). In thiamine deficiency tryptophan may be effectively metabolized via the TP pathway to 3-hydroxyanthranilic acid and it's end products without detectable conversion (paper chromatography) to anthranilic acid or xanthurenic acid for the following reasons: (a) less tryptophan is metabolized to kynurenine owing to factor 2 (above), (b) the kynureninase pathway is relieved of the inhibitory influence of DPT and thiamine and (3) because kynureninase degrades 3-hydroxykynurenine faster than it does kynurenine, kynurenine is probably more rapidly metabolized to 3-hydroxyanthranilic acid than the anthranilic acid (192).

Dalgliesh found that rats deprived of both thiamine and pyridoxine excrete traces of kynurenine, hydroxykynurenine and xanthurenic acid when administered a tryptophan load. Again this finding represents the net result of multiple effects, due to deprivation of both vitamins, some of which are the following: (a) thiamine deficiency increases the amount of tryptophan

being degraded by transamination, as in factor 2(mentioned earlier); (b) thiamine deficiency relieves the endogenous control of kynureninase activity; and (c) pyridoxine deficiency decreases the activity of both kynureninase and transaminase enzymes by depletion of coenzyme. Dalgliesh also states that the administration of a kynurenine load to the doubly deficient rats results in a large output of the metabolites characteristic of pyridoxine deficiency. This finding supports the hypothesis that thiamine deficiency favours the degradation of tryptophan by transamination.

In conclusion thiamine and diphosphothiamine have been shown to be inhibitors of kynureninase in vitro, a finding which may have considerable significance on the control of the activity of this enzyme in vivo. In addition an alternative explanation for the findings of Dalgliesh, on the role of thiamine in tryptophan metabolism, has been suggested in view of the multiple role of this vitamin in intermediary metabolism.

D. TRYPTOPHAN PYRROLASE

i) The Influence of Dietary Factors on Tryptophan Pyrrolase

The administration of tryptophan or certain analogues of tryptophan to animals increases the activity of the tryptophan pyrrolase of the liver. This is attributed to the adaptive nature of this enzyme, although de novo synthesis of enzyme by direct determination of enzyme protein has not been demonstrated so far. This is in part due to the extreme lability of the enzyme in tissue preparations.

The activity of TP is assayed in cell-free tissue preparations thereby

avoiding any influences due to permeability factors. The possibility that the activity of the constitutive level (activity before induction) of TP is restricted by the presence of an inhibitor or that the induced level of TP is the result of the release of an endogenous activator has been excluded by the work of several investigators. Knox and Mehler (156) and Thomson and Mikuta (168) have shown that the activity of a mixed enzyme preparation from control and induced animals is a summation of the individual activities.

The TP activity of rat liver has been shown to be influenced by dietary factors in particular by the level of tryptophan in the diet. Rats fed diets containing 0.2 to 0.9% tryptophan were found to have double the TP activity that rats deprived of tryptophan have. In these experiments the animals were maintained on the diets for twelve to twenty-eight days during which time the disparate TP activites were revealed (Tables XIII and XV). Nevertheless other factors than the dietary tryptophan are responsible for maintaining TP activity in the livers of rats. The relatively low activity in the livers of rats fed Purina chow was not found to be significantly different from that found in the livers of rats deprived of tryptophan. This may be due in part to a lower concentration of tryptophan in the Purina chow than that added to the synthetic diets or to a decreased availability of the amino acid when offered in protein form, or both. On the other hand the TP activity of the rats fed diets from which tryptophan was omitted probably reflects another important factor influencing this enzyme.

While the activity of the enzyme <u>in vivo</u> is related to the availibility

of endogenous tryptophan it is also under hormonal control as evidenced by the decrease in activity following extirpation of the adrenal glands (164, Table XVII) or hypophysis (167). After adrenalectomy or hypophysectomy TP can still be induced by the administration of tryptophan (164, Table $XVII_B$) or certain steroids such as cortisone or hydrocortisone but not by desoxycorticosterone or tetrahydrocortisone (166). It is conceivable that the hormonal effect is due in part to the release of free amino acids in vivo thereby effecting a tryptophan-induced response. On the other hand the possibility that the two mechanisms are distinct and synergistic is suggested by the decreased response of hypophysectomized rats to the administration of tryptophan (168). Although TP is demonstrable late in gestation in fetal guinea pigs an adaptive response is not evoked by the administration of tryptophan nor by the simultaneous administration of tryptophan and ACTH to the fetus or mother. At this stage of development TP activity is not influenced by the availibility of its substrate.

The administration of substances such as adrenaline, histidine or hypertonic saline to intact rats effects an increase in their liver TP activity but does not have this effect in adrenalectomized rats (164,165). This response is considered to be mediated through the adrenal cortex. Similarly deprivation of an essential amino acid is no doubt a stressing influence capable of hormonal stimulation. The TP activity found in rats fed diets from which tryptophan was omitted may then represent a multiple effect: a hormonally evoked increase in TP activity due to the stressing influence of the deficiency

and utilization of endogenous tryptophan stores which may be related in part to hormonal stimulation and in part to release of tryptophan during the normal process of tissue protein degradation. For this reason depriving intact rats of their dietary tryptophan does not give a true representation of their basal TP activities.

Evidence for hormonal stimulation of TP activity as a result of a dietary deficiency has been found in the investigation of the effects of thiamine deficiency on TP. A two-fold increase in the constitutive level (activity before induction) of TP was found in intact rats deprived of exogenous thiamine (Tables XVI and XVII). On the other hand adrenalectomized rats deprived of thiamine did not exhibit this response to the deficiency and had TP levels similar to adrenalectomized control animals (Table XVII, Experiment B). A similar increase in the constitutive level of TP in thiamine deficient intact rats can be observed in the results of other investigators (183,184). The administration of the thiamine antimetabolites, oxythiamine and pyrithiamine, for twelve days to intact rats also resulted in a two-fold increase in the constitutive levels of TP in their livers (Table XVI). These responses too were likely elicited through the adrenal cortex as in addition to their elevated TP activities the rats evinced symptoms of their treatment by changes in body weight and the development of symptoms of polyneuritis (pyrithiamine). Although thiamine deprivation appeared to elevate the constitutive level of TP in intact rats it did not influence the response to tryptophan, and similar levels of TP activity were achieved in these rats after the administration of tryptophan as in control animals (Tables XVI and XVII). In one experiment, however, in which tryptophan made up 0.9% of the

diet thiamine deficiency did not appear to influence the constitutive level of TP (Table XVIII). This apparent discrepancy could be attributed to the high tryptophan content of the diet which in maintaining high levels of TP activity may have effectively concealed any stimulation of activity mediated hormonally.

ii) Conditions Affecting the Activity of Tryptophan Pyrrolase *in vitro*

The possibility that TP is a rapidly metabolized enzyme protein (high turnover) and that what may appear to be adaptation following the administration of tryptophan or some analogues of tryptophan is the result of other factors cannot be overlooked. The effect of administering tryptophan could conceivably result in the stabilization of TP either by combining with it or by inhibiting the action of a protease responsible for the degradation of TP.

One of the features of this enzyme is its instability in liver extracts in the absence of its substrate (39,154,155, Table X, Experiment A), a feature which may reflect the rapid degradation of the enzyme *in vivo*. Dubnoff and Dimick (155) have found that the substrate-induced enzyme is more stable in liver extracts than the enzyme induced by the administration of cortisone. Moreover, they suggest that the greater stability of the former preparation is due to the higher level of tryptophan in the liver tissue following the administration of this amino acid.

When labelled valine is administered to rats and is followed by the administration of tryptophan, a ten-fold increase in tryptophan pyrrolase

activity is found associated with a ten-fold incorporation of the isotope into the liver fraction containing the enzyme (174). The incorporation of the isotope is much higher in this fraction than in the serum proteins. This is strong evidence for the de novo synthesis of enzyme protein but does not exclude the possibility that the newly synthesized protein rather than being degraded is stabilized by the increase in cellular tryptophan. The evidence for the inhibition of tryptophan pyrrolase synthesis by the methionine antimetabolite, ethionine, is not entirely in favour of the adaptation hypothesis. Ethionine has been shown to reduce the adaptive formation of tryptophan pyrrolase of male and female rats administered cortisone (171) or tryptophan (170). However, Farber and Corban (172) find that the administration of ethionine also reduces the constitutive level of tryptophan pyrrolase and inhibits the incorporation of radioactive amino acids into the total liver and plasma proteins in female rats but does not have this action in male rats. Horton and Franz (171) on the other hand have found a reduction of the constitutive level of tryptophan pyrrolase in male rats following thionine administration. Assuming that ethionine interferes with the uptake of methionine thereby interfering with the uptake of other amino acids in protein synthesis, the fact that it also inhibits the constitutive formation of tryptophan pyrrolase within a few hours supports the hypothesis that this enzyme is rapidly metabolized in vivo.

Experiments in vitro have shown that three- to four-fold increases in tryptophan pyrrolase activity can be obtained by perfusing the isolated liver of rat or rabbit with a mixture of blood, casein and supplementary tryptophan (159).

Even greater increases have been reported by Efimochkina (160) who employed a liver slice technique. In her experiments, liver slices were incubated in a medium containing 0.2% glucose and tryptophan (see Fig. 4). In the experiments reported in this thesis increases in TP activity in slices of the order of those found by Efimochkina were not obtained. Glucose was not included in the incubation medium, however the addition of casein hydrolysate and supplementary amino acids or the addition of serum to the medium did not stimulate an increased synthesis of TP (Table XVIII).

It must be kept in mind that tryptophan pyrrolase is not the only enzyme metabolizing tryptophan which increases in activity following the administration of tryptophan to the intact animal. Tryptophan- -ketoglutarate transaminase also increases in activity (36) and may account for as much of the metabolism of tryptophan as the tryptophan pyrrolase system (157). Unfortunately Efimochkina's determinations of the activity of tryptophan pyrrolase are based on tryptophan disappearance rather than kynurenine formation and as a result represent a measure of both the tryptophan pyrrolase and transaminase systems. Civen and Knox (161) have reported briefly that they are able to obtain ever two-fold increases in the tryptophan pyrrolase activity of slices during incubation periods of two to four hours. These increases are presumably based on the determination of kynurenine formation. Occasionally, similar increases were observed in experiments reported in this thesis after one hour's incubation of slices in a medium containing tryptophan. These increases may be interpreted to represent the net effect of enzyme synthesis and substrate stabilization in the tryptophan-induced slices and enzyme synthesis and spontaneous

inactivation in the slices incubated without tryptophan. Although the endogenous activity of slices did not appear to decrease significantly during the first hour of incubation (Fig. 3) the net increase in TP activity of slices incubated with tryptophan only occurred during the first 30 minutes of incubation.

The TP activity of slices incubated with tryptophan and without tryptophan was found to increase during the first 30 minutes although the rate of increase was greater in the slices incubated with tryptophan especially during the first 15 minutes of incubation (Fig. 3). This suggests that the protein synthesis which had occurred in vivo was prolonged for this period of time in the slices. The increases in TP activity can be interpreted as resulting from stabilization of the newly formed enzyme by tryptophan released during the slicing procedure (control slices) or by tryptophan supplied in the incubation medium. After the first 30 minutes, inactivation of the enzyme proceeds at the same rate as synthesis of new enzyme or alternately synthesis of new enzyme stops and the activity of that already formed is stabilized by the presence of substrate.

The greater rate of increase in TP activity which was found to occur during the first 15 minutes in slices incubated with α-methyltryptophan as compared to slices incubated without tryptophan may be the result of (Fig. 3): (1) adaptive formation of enzyme or (2) stabilization of an enzyme having a high turnover, or (3) both. Both α-methyltryptophan and 5-hydroxytryptophan effect increases in the TP activity of slices although both are inhibitors of the enzyme (Table XVII). 5-Hydroxytryptophan is a competitive inhibitor of the bacterial enzyme (153) and it is conceivable that both tryptophan analogues

stabilize the enzyme by combining with it.

Efimochkina has found that marked inhibition of the adaptive formation of TP in slices occurs when 2,4-dinitrophenol is included in the incubation medium. In the present experiments no energy requirement could be demonstrated by incubation of slices under an atmosphere of N_2 or in addition to N_2 by the introduction of a glycolytic poison, iodoacetate, to the medium. It is conceivable that these factors may not have taken effect at the time that TP formation was most actively occurring, during the first 15 minutes of incubation. It is moreover, equally conceivable that the increases in activity were merely reflections of the effect of the availibility of tryptophan on enzyme stabilization. In such a case there would be no demonstrable energy requirement.

Threonine dehydrase is another enzyme whose adaptive formation has been demonstrated in isolated perfused liver. Chytil has found that the simultaneous administration of tryptophan and threonine to animals produces a greater response in TP activity than that resulting from the administration of tryptophan alone. Two mechanisms could be responsible for this effect: (1) a greater mobilization of amino acids than that resulting from the administration of tryptophan alone which are used more effectively for TP formation or (2) the administration of threonine may stimulate TP formation through hormonal routes. Threonine was tested for its possible activating effect of TP formation in slices. No evidence for the first mechanism could be obtained, however due to the uncertainty of actual protein synthesis occurring in the slices, this must be considered inconclusive.

No evidence for the assimilation of new enzyme protein as TP was demonstrated in liver slices, rather it is felt that the increase in TP activity of slices incubated with tryptophan was a result of substrate stabilization of this labile enzyme.

V. SUMMARY

Several divergent pathways of tryptophan metabolism have been investigated by means of nutritional studies and experiments *in vitro*. These pathways are the ones forming and degrading kynurenine and 5-hydroxyindoles, and the pathway utilizing D-tryptophan. The role of thiamine in the metabolism of tryptophan has also been investigated.

1. Deprivation of tryptophan has been found to be reflected in the 5-hydroxytryptamine content of the small intestine and in the excretion of 5-hydroxyindoleacetic acid within 24 hours. Both of these metabolites of tryptophan were reduced to the extent of approximately 50% of their control values within this time. The rapid decrease in the small intestinal content of 5-hydroxytryptamine suggests that at least 50% is replaced every 24 hours. Following this initial decrease in 5-hydroxyindole stores endogenous tryptophan appeared to sustain synthesis along this pathway for at least 21 days at a fairly constant level. The administration of reserpine to tryptophan deprived rats was not found to augment the deficiency during the 21 days that the urinary 5-hydroxyindoleacetic acid was determined. This finding supports those of other investigators that reserpine does not interfere with the biosynthesis of 5-hydroxytryptamine. Approximately 0.1% of the dietary tryptophan has been estimated to be metabolized as 5-hydroxyindoles.

2. The tryptophan pyrrolase pathway forming formylkynurenine has been found to be influenced by the tryptophan content of the diet. This finding is attributed to the adaptive nature of this enzyme.

3. Both the D- and L-isomers of tryptophan have been found to be equally effective in promoting 5-hydroxyindole synthesis and tryptophan pyrrolase activity in the rat. This similarity was found in their ability to promote liver and body growth, confirming the results of earliest investigators. Under anaerobic conditions the tryptophan pyrrolase activity of liver slices was not increased by D-tryptophan although it was increased with L-tryptophan. Under aerobic conditions D-tryptophan was found to promote tryptophan pyrrolase activity in slices but not as effectively as the L-isomer. This finding suggested that the activity of D-tryptophan is dependent on stereo-specific inversion to L-tryptophan, a conversion which could be mediated through D-amino acid oxidase and transamination.

4. The Michaelis constant for kynureninase was found to be 0.67×10^{-3} M using L-kynurenine. This value is somewhat higher than that found by an earlier investigator.

5. Thiamine has been found to be without direct influence on the activity of tryptophan pyrrolase *in vitro* or on the synthesis of this enzyme in intact and adrenalectomized rats. Thiamine has also been found to be without effect on the adaptation mechanism by which tryptophan pyrrolase activity is increased in the liver. In intact rats thiamine deprivation has been found to increase the tryptophan pyrrolase activity of the liver but this effect is attributed to hormonal mediation because it was not found in adrenalectomized rats. The administration of thiamine antimetabolites, oxythiamine and pyrithiamine, to rats was not found to influence the constitutive or induced formation of

tryptophan pyrrolase in any way except that expected through hormonal stimulation.

Both thiamine and diphosphothiamine were found to be inhibitory to the kynureninase system in vitro. This inhibition was not relieved by substrate concentrations below that necessary for half-maximal velocity of the enzyme but was relieved by substrate concentrations above this value. The inhibition of kynureninase by diphosphothiamine has been formally characterized as competitive. Increasing the availibility of the coenzyme pyridoxal phosphate did not relieve this inhibition, although the enzyme preparation was always pre-incubated with the coenzyme before addition of the inhibitor substance.

In view of the inhibition of kynureninase by thiamine and diphosphothiamine, and the known role of thiamine in carbohydrate metabolism, an alternative suggestion has been offered to explain results in the literature which imply that thiamine is involved at the tryptophan pyrrolase stage of tryptophan degradation.

6. Experiments have been described in which the adaptation of tryptophan pyrrolase was investigated in liver slices. Increases in activity of this enzyme were found to occur during the first 30 minute incubation period, but not later. The increase was found in slices incubated with and without tryptophan although a greater increase was found in the presence of tryptophan; it was not affected by anaerobic conditions nor the presence of iodoacetate. The addition of hydrolysed casein, serum of threonine to the incubation medium had no influence in stimulating the activity of tryptophan pyrrolase in slices. The activity was increased by L-tryptophan, α-methyl-DL-tryptophan, 5-hydroxy-DL-tryptophan and D-tryptophan under aerobic conditions. No evidence for

protein accumulation was obtained and the increase in activity of the slices could be largely attributed to stabilization of the labile enzyme by tryptophan or certain analogues of tryptophan.

Claims to original contribution to knowledge

1. Deprivation of tryptophan has been found to influence markedly the 5-hydroxytryptamine content of the small intestine and the urinary 5-hydroxyindoleacetic acid excretion of rats. These 5-hydroxyindoles are decreased to the extent of 50% within 24 hours after which endogenous tryptophan sustains synthesis along this pathway for at least 21 days. Reserpine administration was not found to augment the effect of tryptophan deprivation. The rapid decrease in 5-hydroxytryptamine of the small intestine suggests that at least 50% is replaced every 24 hours. Approximately 0.1% of dietary tryptophan is metabolized as 5-hydroxyindoles by the rat.

2. The D- and L-isomers of tryptophan have been found to be equally effective in promoting 5-hydroxyindole synthesis and tryptophan pyrrolase activity in the intact rat. D-tryptophan was effective in promoting tryptophan pyrrolase activity in liver slices only to the extent that it was converted to the L-isomer.

3. The amount of tryptophan pyrrolase in rat liver, "the constitutive level", is directly influenced by the concentration of tryptophan in the diet.

In confirmation of other investigators' findings, thiamine did not influence the constitutive level of tryptophan pyrrolase in rat liver or the ability of rats to form the enzyme adaptively following a tryptophan load.

Simultaneous thiamine and tryptophan deficiency had no effect upon the constitutive level of tryptophan pyrrolase other than that attributable

to tryptophan deficiency alone. Furthermore there was no associated effect of thiamine deficiency and adrenalectomy on the adaptive formation of tryptophan pyrrolase following the administration of tryptophan.

4. The Michaelis constant for kynureninase has been estimated to be 0.67×10^{-3}M using L-kynurenine. Kynureninase has been found to be inhibited by thiamine and diphosphothiamine *in vitro*. The inhibition is relieved by increasing the substrate concentration above that necessary for half-maximum velocity of the enzyme. The inhibition is not relieved by excess pyridoxal phosphate.

5. Increases in the activity of tryptophan pyrrolase were found to occur in liver slices. This increase was attributed largely to stabilization of the enzyme by the substrate or a closely related analogue. Effective compounds were: DL-tryptophan, L-tryptophan, D-tryptophan (aerobically only), α-methyl-DL-tryptophan, 5-hydroxy-DL-tryptophan.

BIBLIOGRAPHY

1. Adamkiewicz, A. Pflügers Arch. ges. Physiol. 9: 156, 1874.

2. Hopkins, F.G., and Cole, S.W.
Proc. Roy. Soc. 68: 21, 1901.

3. Hopkins, F.G., and Cole, S.W.
J. Physiol. 27: 418, 1901.

4. Hopkins, F.G., and Cole, S.W.
J. Physiol. 29: 451, 1903.

5. Ellinger, A., and Flamand, C.
Ber. chem. Ges. 40: 3029, 1907.

6. Willcock, E.G., and Hopkins, F.G.
J. Physiol. 35: 88, 1906-1907.

7. Mendel, L.B. Harvey Lectures. 10: 101, 1914-1915.

8. Osborne, T.B., and Mendel, L.B.
J. Biol. Chem. 17: 325, 1914.

9. Rose, W.C. Science 86: 298, 1937.

10. Rose, W.C., Oesterling, M.J., and Womack, M.
J. Biol. Chem. 176: 753, 1948.

11. Williams, H.H., Curtin, L.V., Abraham, J., Loosli, J.K., and Maynard, L.A.
J. Biol. Chem. 208: 277, 1954.

12. Berg, C.P., and Potgieter, M.
J. Biol. Chem. 94: 661, 1931-1932.

13. du Vigneaud, V., Sealock, R.R., and van Etten, C.
J. Biol. Chem. 98: 565, 1932.

14. Berg, C.P. J. Biol. Chem. 104: 373, 1934.

15. Berg, C.P., Rose, W.C., and Marvel, C.S.
J. Biol. Chem. 85: 207, 1929.

16. Gordon, W.E., and Jackson, R.W.
J. Biol. Chem. 110: 151, 1935.

17. Cahill, W.M., and Kotalik, G.C.
J. Nutrition 26: 471, 1943.

18. Berg, C.P., Rose, W.C., and Marvel, C.S.
J. Biol. Chem. 85: 219, 1929.

19. Baugess, L.C., and Berg, C.P.
J. Biol. Chem. 104: 675, 1934.

20. Berg, C.P., and Baugess, L.C.
J. Biol. Chem. 98: 171, 1932.

21. Totter, J.R., and Day, P.L.
J. Nutrition 24: 159, 1942.

22. Albanese, A.A., and Buschke, W.
Science 95: 584, 1942.

23. Albanese, A.A., Randall, R.M., and Holt, L.E., Jr.
Science 97: 312, 1943.

24. Goldberger, J., Waring, C.H., and Willets, D.G.
U.S. Public Health Repts. 30: 3117, 1915.

25. Goldberger, J., and Tanner, W.F.
U.S. Public Health Repts. 37: 462, 1922.

26. Weissbach, H., King, W., Sjoerdsma, A., and Udenfriend, S.
J. Biol. Chem. 234: 81, 1959.

27. Armstrong, M.D., Shaw, K.N.F., Gortatowski, M.J., and Singer, H.
J. Biol. Chem. 232: 17, 1958.

28. Udenfriend, S., and Titus, E.
A Symposium on Amino Acid Metabolism, edited by McElroy, W.D., and Glass, H.B., The Johns Hopkins Press, Baltimore, Md. 1955. p. 945.

29. Udenfriend, S., Weissbach, H., and Sjoerdsma, A.
Science 123: 669, 1956.

30. Price, J.M., Brown, R.R., and Ellis, M.E.
J. Nutrition 60: 323, 1956.

31. Schayer, R.W. J. Biol. Chem. 187: 777, 1950.

32. Gholson, R.K., Rao, D.R., Henderson, L.M., Hill, R.J., and Koeppe, R.E.
J. Biol. Chem. 230: 179, 1958.

33. Hankes, L.V., and Henderson, L.M.
J. Biol. Chem. 225: 349, 1957.

34. Keller, C., and Lang, K.
Biochem. Z. 328: 285, 1956.

35. Lin, E.C.C., Pitt, B.M., Civen, M., and Knox, W.E.
J. Biol. Chem. 233: 668, 1958.

36. Lin, E. C.C., Civen, M., and Knox, W.E.
J. Biol. Chem. 233: 1183, 1958.

37. Kotake, Y., and Iwao, J.
Z. physiol. Chem. 195: 139, 1931.

38. Kotake, Y., and Masayama, T.
Z. physiol. Chem. 243: 237, 1936.

39. Knox, W.E., and Mehler, A.H.
J. Biol. Chem. 187: 419, 1950.

40. Mehler, A.H., and Knox, W.E.
J. Biol. Chem. 187: 431, 1950.

41. Knox, W.E. Personal communication, 1959.

42. Liebig, J. Ann. Chem. 86: 125, 1853.

43. Ellinger, A. Z. physiol. Chem. 43: 325, 1904.

44. Ellinger, A., and Matsuoka, Z.
Z. physiol. Chem. 109; 259, 1920.

45. Baugess, L.C., and Berg, C.P.
J. Biol. Chem. 104: 691, 1934.

46. Matsuoka, Z., Takemura, S., and Yoshimatsu, N.
Z. physiol. Chem. 143: 199, 1925.

47. Borchers, R., Berg, C.P., and Whitman, N.E.
J. Biol. Chem. 145: 657, 1942.

48. Kotake, Y., and Nakayama,T.
Z. physiol. Chem. 270: 76, 1941

49. Brown, R.R., and Price, J.M.
J. Biol.Chem. 219: 985, 1956.

50. Wiss, O. Z. physiol. Chem. 293: 106, 1953.

51. Wiss, O. Z. Naturforsh. 7B: 133, 1952.

52. Heidelberger, C., Gullberg, M.E., Morgan, A. F., and Lepkovsky, S.
J. Biol. Chem. 179: 143, 1949.

53. Kotake, Y., Shibata, Y., and Toratani, A.
Proc. Japan Academy 32: 774, 1956.

54. de Castro, F.T., Price, J.M., and Brown, R.R.
J. Amer. Chem. Soc. 78: 2904, 1956.

55. Saito, Y., Hayaishi, O., and Rothberg, S.
J. Biol. Chem. 229: 921, 1957.

56. Braunstein, A.E., Goryochenkova, E.V., and Paskhina, T.S.
Biokhimiya 14: 163, 1949.

57. Musajo, L. Atti. accad. Lincei 21: 368, 461, 1935.
Musajo, L., and Chiancone, F.M.
Atti. accad. Lincei 21: 468, 1935,
as seen in Chem. Abst. 29: 7347, 1935.

58. Lepkovsky, S., Roboz, E., and Haagen-Smith, A.J.
J. Biol.Chem. 149: 195, 1943.

59. Makino, K.,and Arai, K.
Science 121: 143, 1955.

60. Mason, M. J. Biol. Chem. 201: 513, 1953.

61. Charconnet-Harding, F., Dalgliesh, C. E.,and Neuberger,A.
Biochem. J. 53: 513, 1953.

62. Henderson, L.M., Koski, R.E., and D'Angeli, F.
J. Biol. Chem. 215: 369, 1955.

63. Hellmann, H., and Wiss, O.
Helv. Physiol. et Pharmacol. Acta 10: C16, 1952.

64. Wiss, O., and Hellmann, H.
Helv. Physiol. et Pharmacol. Acta 10: C35, 1952.

65. Wiss, O., and Hellmann, H.
Z. Naturforsch, 8b: 70, 1953.

66. Dalgliesh, C., Knox, W.E., and Neuberger, A.
Nature 168: 20, 1951.

67. Dalgliesh, C.E. Biochem. J. 52: 3, 1952.

68. Krehl, W.A., Teply, L.J., Sarma, P.S., and Elvehjem, C.A.
Science 101: 489, 1945.

69. Heidelberger, C., Abraham, E.P., and Lepkovsky, S.
J. Biol. Chem. 179: 151, 1949.

70. Hankes, L.V., and Urivetsky, M.
Arch. Biochem.Biophysics 52: 484, 1954.

71. Miyake, A., Bokman, A.H., and Schweigert, B.S.
J. Biol. Chem. 211: 391, 1954.

72. Bokman, A.H., and Schweigert, B.S.
Arch. Biochem. Biophysics 33: 270, 1951.

73. Wiss, O. Z. physiol. Chem. 304: 221, 1956.

74. Wiss, O., and Bettendorf, G.
Z. physiol. Chem. 306: 145, 1957.

75. Henderson, L.M., and Ramasarma, G.B.
J. Biol. Chem. 181: 687, 1949.

76. Bokman, A.H., and Schweigert, B.S.
J. Biol. Chem. 186: 153, 1950.

77. Long, C.L., Hill, H.N., Weinstock, I.M., and Henderson, L.M.
J. Biol. Chem. 211: 405, 1954.

78. Mehler, A.H. J. Biol. Chem. 218: 241, 1956.

79. Mehler, A.H., and May, E.L.
J. Biol. Chem. 223: 449, 1956.

80. Suhadolnik, R.J., Stevens,C.O., Decker,R.H., Henderson, L.M., and Hankes, L.V.
J. Biol. Chem. 228: 973, 1957.

81. Hankes, L.V., and Segal, I.H.
Proc. Soc. Exp. Biol. Med. 94: 447, 1957.

82. Henderson, L.M. J. Biol.Chem. 181: 677, 1949.

83. Ellinger, P. Biochem. J. 42: 175, 1948.

84. Cantoni, G. J. Biol. Chem. 189: 203, 1951.

85. Knox, W.E., and Grossman, W.I.
J. Biol. Chem. 166: 391, 1946.

86; Knox, W.E., and Grossman, W.I.
J. Biol. Chem. 168: 363, 1947.

87. Hunter, S.F., and Handler, P.
Arch. Biochem. Biophysics 35: 377, 1952.

88. Erspamer, V. Arch. Exp. Path. u. Pharmak. 200: 60, 1942.

89. Erspamer, V. Experientia 2: 369, 1946.

90. Erspamer, V. Acta Pharmacol. 4: 213, 1948.

91. Erspamer, V., and Boretti, G.
Arch. int. Pharmacodyn. 88: 296, 1951.

92. Erspamer, V., and Asero, B.
Nature 169: 800, 1952.

93. Erspamer, V. Pharmacol. Rev. 6: 425, 1954.

94. Rapport, M.M., Green, A.A., and Page, I.H.
Fed. Proc. 6: 184, 1947.

95. Rapport, M.M., Green, A.A., and Page, I.H.
J. Biol. Chem. 174: 735, 1948.

96. Rapport, M.M., Green, A.A., and Page, I.H.
Science 108: 329, 1948.

97. Rapport, M.M., Green, A.A., and Page, I.H.
J. Biol. Chem. 176: 1243, 1948.

98. Rapport, M.M. J. Biol. Chem. 180: 961, 1949.

99. Hamlin, K.E., and Fisher, F.E.
J. Amer. Chem. Soc. 73: 5007, 1951.

100. Speeter, M.E., Heinzelmann, R.V., and Weisblat, D.I.
J. Am. Chem. Soc. 73: 5515, 1951.

101. Asero, B., Colo, V., Erspamer, V., and Vercellone, A.
Ann. Chem. 576: 69, 1952.

102. Ek, A., and Witkop, B.
J. Am. Chem. Soc. 75: 500, 1953.

103. Udenfriend, S., Clark, C.T., and Titus, E.
Fed. Proc. 12: 282, 1953.

104. Dalgliesh, C.E., and Dutton, R.W.
Brit. J. Cancer 11: 296, 1957.

105. Eber, O., and Lembeck, F.
Arch. ges. Physiol. 265: 563, 1958.

106. Sjoerdsma, A., and Udenfriend, S.
J. Clin, Invest. 34: 914, 1955.

107a. Udenfriend, S., Weissbach, H.
Fed. Proc. 13: 412, 1954.

107b. Udenfriend, S., Titus, E., Weissbach, H., and Peterson, R.E.
J. Biol. Chem. 219: 335, 1956.

108. Udenfriend, S., and Weissbach, H.
Proc. Soc. Exp. Biol. Med. 97: 748, 1958.

109. Sandler, M., and Snow, P.J.D.
Lancet 7012: 137, 1958.

110. Lembeck, F. Nature 172: 910, 1953.

111. Giarman, N.J. Fed. Proc. 15: 428, 1956.

112. Gaddum, J.H., and Giarman, N.J.
Brit. J. Pharmacol. 11: 88, 1956.

113. Udenfriend, S., Clark, C.T., and Titus, E.
J. Am. Chem. Soc. 75: 501, 1953.

114. Clark, C.T., Weissbach, H., and Udenfriend, S.
J. Biol. Chem. 210: 139, 1954.

115. Beiler, J.M., and Martin, G.J.
J. Biol. Chem. 211: 39, 1954.

116. Buzard, J.A., and Nytch, P.D.
J. Biol. Chem. 229: 409, 1957.

117. Udenfriend, S., Weissbach, H., and Bogdanski, D.F.
Ann. N.Y. Acad. Sci. 66: 602, 1957.

118. Bogdanski, D.F., Weissbach, H., and Udenfriend,S.
Fed. Proc. 15: 402, 1956.

119. Udenfriend, S., Bogdanski, D.F., and Weissbach, H.
Fed. Proc. 15: 493, 1956.

120. Freter, K., Weissbach, H., Udenfriend, S., and Witkop, B.
Proc. Soc. Exp. Biol. Med. 94: 725, 1957.

121. Davidson, J., Sjoerdsma, A., Loomis, L.N., and Udenfriend, S.
J.Clin, Invest. 36: 1594, 1957.

122. Udenfriend, S., Weissbach, H., and Bogdanski, D.F.
J. Biol.Chem. 224: 803, 1957.

123. Giarman, N.J., Green, V.S., Green, J.P., and Paasonen, M.K.
Proc. Soc. Exp. Biol. Med. 94: 761, 1957.

124. Feldberg, W., and Toh, C.C.
J. Physiol. 119: 352, 1953.

125. Twarog, B.M., and Page, I.H.
Amer. J. Physiol. 175: 157, 1953.

126. Amin, A.H., Crawford, T.B.B., and Gaddum, J.H.
J. Physiol. 126: 596, 1954.

127. Correale, P. J. Neurochem. 1: 22, 1956.

128. Bogdanski, D.F., Weissbach, H., and Udenfriend, S.
J. Neurochem. 1: 272, 1957.

129. Zucker, M.B., and Rapport, M.M.
Fed. Proc. 13: 170, 1954.

130. Titus, E., and Udenfriend, S.
Fed. Proc. 13: 411, 1954.

131. Erspamer, V. Arch. exp. Path. u. Pharmak. 200: 43, 1942.

132. Erspamer, V. Arch. exp. Path. u. Pharmak. 201: 377, 1943.

133. Bogdanski, D.F., and Udenfriend, S.
J. Pharmocol. Expt. Therap. 116: 7, 1956.

134. Erspamer, V. Experientia 10: 471, 1954.

135. Erspamer, V. J. Physiol. 127: 118, 1955.

136. Weissbach, H., Redfield, B.G., and Udenfriend, S.
Fed. Proc. 17: 418, 1958.

137. McIsaac, W.M., and Page, I.H.
Science 128: 537, 1958.

138. Pletscher, A., Shore, P.A., and Brodie, B.B.
Science 122: 374, 1955.

139. Brodie, B.B., Pletcher, A., and Shore, P.A.
Science 122: 968, 1955.

140. Shore, P.A., Carlsson, A., and Brodie, B.B.
Fed. Proc. 15: 483, 1956.

141. Green, J.P., Paasonen, M.K., and Giarman, N.J.
Proc. Soc. Exp. Biol. Med. 94: 428, 1957.

142. Hess, S.M., Shore, P.A., and Brodie, B.B.
Fed. Proc. 15: 437, 1956.

143. Kuntzman, R., Udenfriend, S., Tomich, E.G., Brodie, B.B., and Shore, P.A.
Fed. Proc. 15: 450, 1956.

144. Erspamer, V., and Ciceri, C.
Experientia 13: 87, 1957.

145. Brodie, B.B., Tomich, E.G., Kuntzman, R., and Shore, P.A.
J. Pharmacol. Expt. Therap. 119: 461, 1957.

146. Shore, P.A., Pletscher, A., Tomich, E.G., Carlsson, A., Kuntzman, R., and Brodie, B.B.
Ann. N.Y. Acad. Sci. 66: 609, 1957.

147. Knox, W.E. Biochim. et Biophys. Acta 14: 117, 1954.

148. Tanaka, T., and Knox, W.E.
Fed. Proc. 17: 320, 1958.

149. Itagaki, C., and Nakayama, Y.
Z. physiol. Chem. 270: 83, 1941.

150. Hayaishi, O., Rothberg, S., Mehler, A.H., and Saito, Y.
J. Biol. Chem. 229: 889, 1957.

151. Sakan, T., and Hayaishi, O.
J. Biol. Chem. 186: 177, 1950.

152. Mason, M., and Berg,C.P.
J. Biol. Chem. 188; 783, 1951.

153. Hayaishi, O. A Symposium on Amino Acid Metabolism, edited by McElroy, W.D., and Glass, H.B., The Johns Hopkins Press, Baltimore, Md., 1955. p. 914.

154. Sourkes, T.L., and Townsend, E.
Can. J. Biochem. Physiol. 33: 735, 1955.

155. Dubnoff, J.W., and Dimick, M.
Biocim. et Biophys. Acta 31: 541, 1959.

156. Knox, W.E., and Mehler, A.H.
Science 113: 237, 1951.

157. Lee, N.D. J. Biol. Chem. 219: 211, 1956.

158. Schor, J.M., and Frieden, E.
J. Biol. Chem. 233: 612, 1958.

159. Price, J.B., Jr.,and Dietrich, L.S.
J. Biol. Chem. 227: 633, 1957.

160. Efimochkina, E.F. Biokhimiya 19: 68, 1954.

161. Civen, M., and Knox, W.E.
Fed. Proc. 16: 165, 1957.

162. Nemeth, A.M., and Nachmias, V.T.
Science 128: 1085, 1958.

163. Auerbach, V.H., and Waisman, H.A.
J. Biol. Chem. 234: 304, 1959.

164. Knox, W.E. Brit. J. Exp. Path. 32: 462, 1951.

165. Knox, W.E., and Auerbach, V.H.
J. Biol. Chem. 214: 307, 1955.

166. Thomson, J.F., and Mikuta, E.T.
Endocrinology 55: 232, 1954.

167. Geschwind, I.I., and Li, C.H.
J. Clin. Endoc. Metab. 14: 789, 1954.

168. Thomson, J.F., and Mikuta, E.T.
Proc. Soc. Exp. Biol. Med. 85: 29, 1954.

169. Pitot, H.C., Dent, J.H., and Feary, T.
Fed. Proc. 17: 290, 1958.

170. Lee, N.D., and Williams, R.H.
Biochim. et Biophys. Acta 9: 698, 1952.

171. Horton, H.R., and Franz, J.M.
Endocrinology 64: 258, 1959.

172. Farber, E., and Corban, M.S.
J. Biol.Chem. 233: 625, 1958.

173. Lee, N.D., and Williams, R.H.
J. Biol. Chem. 204: 477, 1953.

174. Gros, P., Talwar, G.P., and Coursaget, J.
Bull. soc. chim. biol. 36: 1569, 1954.

175. Sayre, F.W., Jensen, D., and Greenberg, D.M.
J. Biol. Chem. 219: 111, 1956.

176. Chytil, F. Abstracts of the Fourth International Congress of Biochemistry, Vienna, 1958.

177. Feigelson, P., Feigelson, M., and Fancher,C.
Fed. Proc. 17: 218, 1958.

178. Lee, N.D. Fed. Proc. 17: 262, 1958.

179. Junqueira, A., and Schweigert, B.S.
J. Biol. Chem. 175: 535, 1948.

180. Dalgliesh, C.E. Biochim. et Biophys. Acta 15: 295, 1954.

181. Dalgliesh, C.E. Biochem. J. 61: 328, 1955.

182. Porter,C.C., Clark, I., and Silber, R.H.
Arch. Biochem. 18: 339, 1948.

183. Ginoulhiac, E. Boll. soc. ital. biol. sper. 32: 1178, 1956.

184. Mangoni, A., Paolucci, A.M., and Spadoni, M.A.
Quaderni nutriz. 17: 38, 1957 as seen in
Chem. Abstracts 52; 18720c, 1958.

185. Tenconi, L.T. Boll. soc. ital. biol. sper. 32: 1057, 1956.

186. Ginoulhiac, E., and Tenconi, L.T.
Acta Vitaminologica 10: 169, 1956.

187. Mason, M., and Berg, C.P.
J. Biol. Chem. 195: 515, 1952.

188. Dalgliesh, C.E. Quart. Revs. (London) 5: 227, 1951.

189. Knox, W.E. Biochem. J. 53: 379, 1953.

190. Wiss, O., and Weber,F.
Z. physiol. Chem. 304: 232, 1956.

191. Wiss, O., and Weber, F.
Z. physiol. Chem. 304: 241, 1956.

192. Wiss, O., and Fuchs, H.
Experientia 6: 472, 1950.

193. Hayaishi, O., and Stanier, R.Y.
J. Biol. Chem. 195: 735, 1952.

194. Jakoby, W.B., and Bonner, D.M.
J. Biol. Chem. 205: 699, 1953.

195. Jakoby, W.B., and Bonner,D.M.
J. Biol. Chem. 205: 709, 1953.

196. Longenecker, J.B., and Snell, E.E.
J. Biol. Chem. 213: 229, 1955.

197. Fisher,R.A., and Yates, F.
Statistical Tables for Biological, Agricultural
and Medical Research, Oliver and Boyd (London),
1948. p. 26, 104-109.

198. Udenfriend, S., Weissbach, H., and Clark, C.T.
J. Biol. Chem. 215: 337, 1955.

199. Weissbach, H., Waalkes, T.P., and Udenfriend, S.
J. Biol. Chem. 222: 865, 1958.

200. Udenfriend, S., Titus, E., and Weissbach, H.
J. Biol. Chem. 216: 499, 1955.

201. MacFarlane, P.S., Dalgliesh, C.E., Dutton, R.W., Lennox, B., Nyhus, L.M., and Smith, A.N.
Scot. Med. J. 1: 148, 1956.

202. Knox, W.E. Methods in Enzymology - Vol. II, edited by Colowick, S.P., and Kaplan, N. O., Academic Press Inc. (New York), 1955. p. 242.

203. Bratton, A.C., and Marshall, E.K., Jr.
J. Biol. Chem. 128: 537, 1939.

204. Umbreit, W.W., Burris, R.H., and Stauffer, J.F.
Manometric Techniques and Tissue Metabolism, Burgess Publishing Co. (Minneapolis), 6th printing 1951. p. 118.

205. Shore, P.A., Silver, S.L., and Brodie, B.B.
Science 122: 284, 1955.

206. Brodie, B.B., and Shore, P.A.
Ann. N.Y. Acad. Sci. 66: 631, 1957.

207. Deming, Q., Bogdanski, D.F., Udenfriend, S., Shore, P.A., and Brodie, B.B.
Fed. Proc. 15: 416, 1956.

208. Furuta, K. Osaka Daig. Igaku Zassi 8: 131, 1956, as seen in Chem. Abstracts 50: 11459f, 1956.

209. Snedecor, G.W. Statistical Methods, Iowa State College Press (Ames, Iowa), 4th Edition, 1946. chapters 10, 13.

210. Wolfe, S.J. J. Biol. Chem. 229: 801, 1957.

211. Lineweaver, H., and Burk, D.
J. Amer. Chem. Soc. 56: 658, 1934.

212. Hearon, J.Z., Bernhard, S.A., Friess, S.L., Botts, D.J., and Morales, M.F.
The Enzymes - Vol. I, edited by Boyer, P.D., Lardy, H., and Myrback, K., Academic Press Inc. (New York), 1959. p. 102.

213. Bogdanski, D.F., Pletscher, A., Brodie, B.B., and Udenfriend, S.
J. Pharmacol. Expt. Therap. 117: 82, 1956.

214. Bicknell, F., and Prescott, F.
The Vitamins in Medicine, edited by Grune, [illegible]
Stratton, (New York), 1953.

215. Barron, E.S.G., Goldinger, J.M., Lipton, M.A., and Lyman, C.M.
J. Biol. Chem. 141: 975, 1941.

216. Green, D.E., Westerfield, W.W., Vennesland, B., and Know, W.E.
J. Biol. Chem. 145: 69, 1942.

217. Ferrari, V. Acta Vitamin. 11: 97, 1957.