Acceleration and Inhibition of Tryptophan Metabolism

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ACCELERATION AND INHIBITION OF TRYPTOPHAN METABOLISM

By

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PREFACE

Tryptophan is functionally important in biological systems, as the free amino acid, as a component of proteins, and as a precursor of physiologically active substances.

If administered in excess amounts, it modifies carbohydrate and fat metabolism and the activities of some enzymes. A dietary deficiency of this essential amino acid results in attenuated growth, and alterations in the metabolism of certain substances. Within the protein molecule, tryptophan is believed to play a role as an intermediate in some enzymic reactions.

Enzymic modifications of this substance give rise to serotonin, a possible neurohumoral agent, and melatonin, a proposed mediator of a diurnal rhythm in some species. An alternate pathway leads to the ultimate formation of NAD, a cofactor of many enzyme systems. 3-Hydroxyanthranilic acid, a metabolic product of tryptophan is converted to eye pigments in insects.

It seems evident, therefore, that the availability of tryptophan is an important factor in the maintenance of biological equilibria.

The enzyme now generally recognized as the most important regulator of tryptophan levels in many organisms is tryptophan pyrrolase; it catalyzes the initial reaction of the pathway which leads to ultimate deamination and total

oxidation of tryptophan.

This thesis has set out to study the factors which increase or decrease the activity of this enzyme, and the role of this pathway. Tryptophan pyrrolase activity can be induced in mammalian liver by giving an excess of tryptophan or corticosteroids. The only other substance known to have this specific effect is α-methyltryptophan. (It should be stated that in the context of this thesis induction implies increased enzyme activity without reference to mechanism.) Sankoff and Sourkes showed that the α-methylamino acid, in contrast to tryptophan causes a very prolonged induction of tryptophan pyrrolase in the rat. It was of interest to determine whether this prolonged effect was due to the continued presence of the inducing agent in the body, or to some other mechanism. The significance of a high pyrrolase activity on endogenous tryptophan was also studied.

Since this amino acid analogue was identified as an inducing agent, it has been used by numerous groups to study the mechanism and significance of the induction. The usefulness of α-methyltryptophan stimulated attempts to uncover other accelerators of tryptophan breakdown. Tests of a large group of indoles and other aromatic substances in vivo have led to the finding that many aromatic derivatives inhibit tryptophan oxidation in vivo. In an attempt to localize the site of inhibition, the effects of selected inhibitors on the oxidation of tryptophan labeled with ^{14}C in different

positions was studied. These results were correlated with their action on tryptophan pyrrolase *in vitro*. Inhibition of tryptophan degradation may be an important tool in uncovering other functions of this pathway and of tryptophan itself.

It was important to investigate the regulation of tryptophan oxidation by substances indigenous to the rat. Thus the influence of various tryptophan metabolites upon pyrrolase was explored *in vivo* because of reports that this enzyme is inhibited by some of these products *in vitro*. Copper has been implicated as a cofactor of the bacterial enzyme and therefore tryptophan pyrrolase activity was measured in the copper-deficient animal. The effect of vitamin B_6-deficiency on the oxidation of labeled tryptophan was studied *in vivo* to assess earlier reports that the activity of kynureninase was reduced in this deficiency state, and that this reduction is responsible for the increased urinary excretion of xanthurenic acid.

In the following section a review of the literature of tryptophan metabolism, tryptophan pyrrolase, and the drugs used in this investigation, is given.

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Mr. K. Lloyd prepared the copper-deficient rats and Mr. G. Gregoriadis analyzed tissue copper.

Miss M.A. Mongeau typed this manuscript with skillfulness and efficiency.

To my husband, I express my appreciation for his constant encouragement, support and valuable advice.

TABLE OF CONTENTS

Page

I. INTRODUCTION

A. General

Tryptophan was first isolated from a pancreatic digest of casein by Hopkins and Cole (1) in 1901. The structure was established by Ellinger and Flamand in 1907 (2).

The amino acid and its derivatives are distributed widely in bacteria and plants, where tryptophan is synthesized. It is essential to animals (3). As some mammals can convert the D- to the L- form, D-tryptophan may serve to maintain nitrogen balance (4). In comparison with most amino acids, it occurs free to a small extent in tissues (5) and in proteins (6). In plasma it circulates in the free state or may be associated with plasma albumin (7).

B. Metabolic Effects of Tryptophan:

Quantitative alterations in dietary tryptophan can generate metabolic changes. Although some of the effects are attributed to tryptophan itself further studies may reveal a more indirect action, possibly owing to the metabolites of tryptophan.

A diet supplemented with excess tryptophan and methionine results in abnormal fat deposition in the liver (8). Administration of tryptophan alone causes a depletion of liver glycogen (9) and induction of at least five enzymes: tryptophan pyrrolase (10), tyrosine transaminase (11), threonine dehydrase (12), ornithine δ-transaminase (12) and phosphoenolpyruvic carboxykinase (9).

A dietary deficiency of tryptophan results in attenuated growth, concomitant with a reduction in the activities of certain enzymes (13, 14).

Another property of tryptophan, which may gain biological significance, is its ability to bind heavy metals, including copper (15) and zinc (16).

Abnormal tryptophan metabolism is associated with several diseases including pellagra, Hartnup's disease, and phenylketonuria (17).

C. Metabolism of Tryptophan

While interest in the metabolism of tryptophan dates back to the beginning of the century one can trace the contemporary concern in this subject to no less than 4 significant discoveries. These are: 1. the identification of the first of the metabolites of tryptophan, kynurenic acid (18); 2. the ability of tryptophan to substitute for niacin in the diet (19); 3. the isolation of serotonin and elucidation of its metabolic relation to tryptophan (20); 4. the inducibility of tryptophan pyrrolase, the enzyme that degrades tryptophan, by tryptophan (10) or glucocorticoids (21).

Exogenous tryptophan is metabolized very rapidly in the body. Within 6 hours of injection of a large dose of tryptophan, no excess tryptophan can be detected in the liver or blood (22); within 24 hours approximately 25% of benzene-labeled tryptophan-^{14}C can be accounted for in respiratory

$^{14}CO_2$ and nearly 40% in protein and lipid (23).

It is now accepted that there are two general routes by which free tryptophan is degraded in tissues; one is concerned with the oxidation of the side-chain, (and/or substitutions on the benzene ring), the other, with the oxidation of the pyrrole ring and benzene ring, resulting in acyclic intermediates which either recyclize or are totally oxidized to CO_2. Both pathways lead to the formation of physiologically active substances.

1. Side Chain Oxidation (Fig. 1)

The oxidation of the side chain accounts for a small percentage of tryptophan that is metabolized (24) although as much as 10% of administered tryptophan has been recovered as indole-3-acetic acid in vitamin B_6-deficiency (25).

a. Tryptamine Pathway

Direct decarboxylation of the side chain yields tryptamine (24,26). The enzyme performing this oxidation is believed to be non-specific, acting on many aromatic compounds (26). Monoamine oxidase (MAO) rapidly converts tryptamine to the aldehyde which is then either oxidized to indole-3-acetic acid (24, 27, 28), or may be reduced to tryptophol (29). Inhibition of MAO results in a large increase in tissue content of tryptamine (30) accompanied by central nervous system effects (31). A major source of urinary indole-3-acetic acid is indolepyruvic acid, formed by transamination of tryptophan (24). Liver microsomes can hydroxylate tryptamine in the 6-position in vitro but this reaction

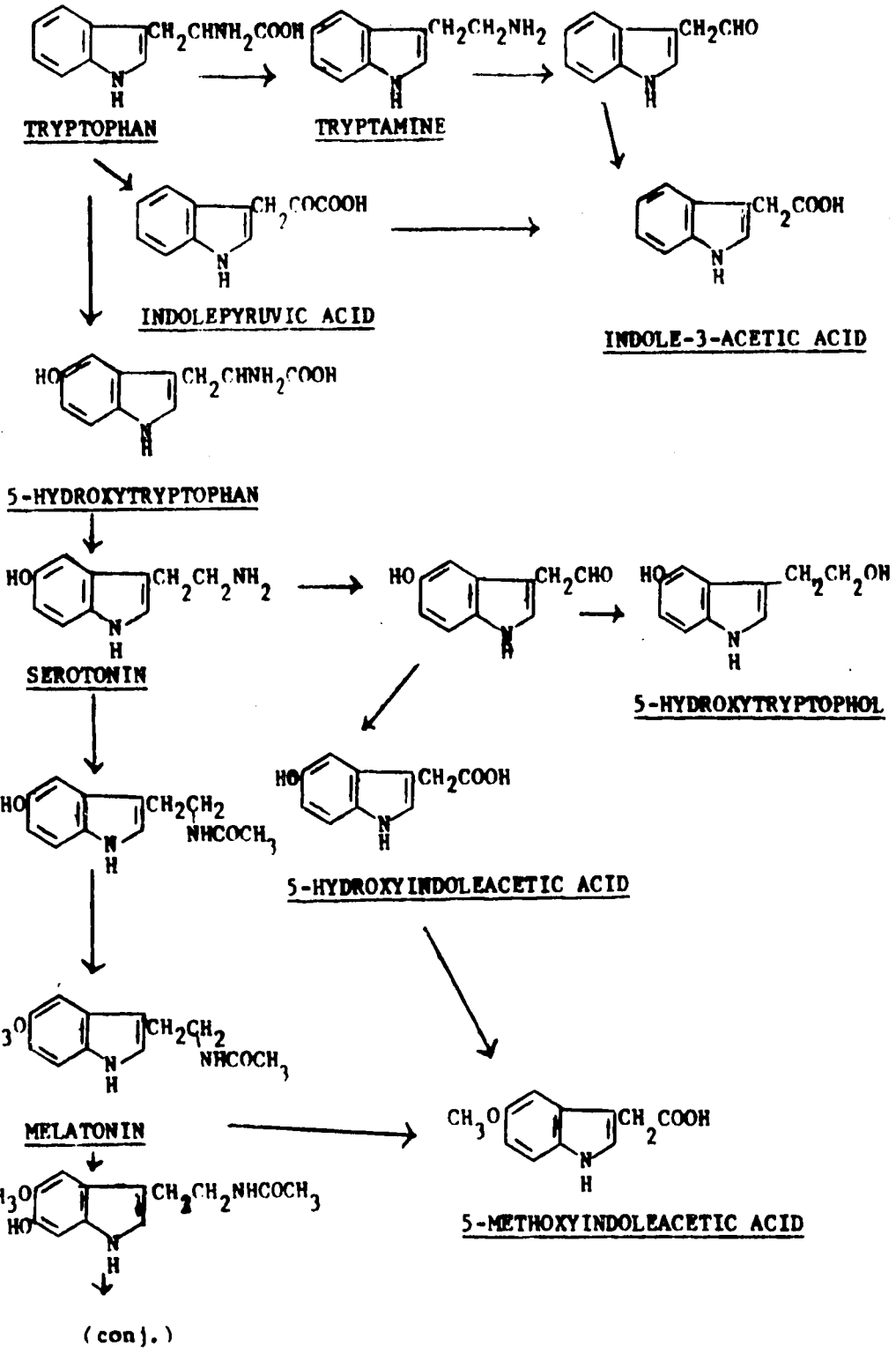


Fig. 1. Metabolism of tryptophan along pathways in which the indole ring remains intact

is probably not of physiological importance (32).

b. Serotonin Pathway

The high biological activity of serotonin (33), its structural analogy to hallucinogenic agents (L.S.D., N-substituted tryptamines) and its function as a presursor of melatonin (34) has made it an object of intensive investigation recently. Serotonin was isolated and identified by Rapport et al. (35) and found to be identical with the enteramine of Erspamer and Ottolenghi. Udenfriend et al. showed that tryptophan was a precursor of serotonin and proposed the pathway for its formation and subsequent metbolism (20). Tryptophan is first hydroxylated to 5-hydroxytryptophan, the rate-limiting enzyme in the formation of serotonin (36). Tryptamine cannot substitute as a substrate (20). The enzyme, previously believed to occur only in peripheral tissues (36), has recently been found in the brain (37, 38), its distribution corresponding to that of serotonin (37). 5-Hydroxytryptophan is decarboxylated by aromatic amino acid decarboxylase (39), a vitamin B_6-enzyme (26). Monoamine oxidase acts rapidly on serotonin (20). Because of the discrepancy between the serotonin metabolized and the 5-hydroxyindoleacetic acid recovered, Wiseman and Sourkes suggested that the aldehyde may undergo other reactions, such as reduction (40). The conversion of serotonin to

5-hydroxytryptophol has been demonstrated (41, 42). In the pineal gland the alcohol is O-methylated by hydroxyindole-o-methyltransferase (43), the product being a regulator of estrus and ovarian growth (44). Another pathway for serotonin is the formation of melatonin (N-acetyl-5-methoxytryptamine) via N-acetylation in the pineal gland (45 -47). Melatonin has been implicated as a regulator of sleep (48) and diurnal biological rhythms (49, 50).

One of the interesting problems relating to this thesis is whether fluctuations in available tryptophan can alter the amount of serotonin produced in vivo. It has recently been shown that animals placed on a diet deficient in tryptophan produce less brain serotonin as early as 4 days after the deficiency (51). A diet containing 5% tryptophan results in 250% increase in the serotonin of the pineal gland and a 100% increase of the amine in the hypothalamus within 5 days (52). In an acute experiment, L-tryptophan, (75 mg/kg or more), given with an MAO inhibitor, leads to a substantial increase in brain serotonin (53). These results indicate that, under appropriate experimental conditions, brain levels of serotonin can be altered considerably by the availability of tryptophan. It is interesting to speculate on the effects that a prolonged induction of tryptophan pyrrolase by substances other than its substrate would have on the production of serotonin.

2. Ring Oxidation of Tryptophan (Fig. 2)

The serotonin pathway, as stated previously accounts for a small percentage of the tryptophan that is metabolized. A major part of tryptophan present in tissues undergoes oxidation of the pyrrole ring. The enzyme which initiates the breakdown, tryptophan pyrrolase, is an inducible enzyme. It is the only identified enzyme that can split the indole ring and as a large proportion of tryptophan is oxidized to CO_2 (23) it is generally accepted that this pathway is the important one regulating the levels of tryptophan in an organism. The aromatic product formed from the pyrrolase reaction enters two metabolic routes of importance: the NAD pathway and the glutarate pathway, after undergoing stepwise oxidation.

a. Conversion to 3-Hydroxyanthranilic Acid

Kynurenic acid was first isolated in urine in 1853 by Liebig (54). It was not until Ellinger (18) discovered increased amounts of the acid in urine of dogs administered tryptophan, that a metabolic relationship between these two substances was postulated. The conversion of tryptophan to kynurenic acid was considered of primary importance in the metabolism of tryptophan until Homer (55) finding this quinaldic acid excreted unchanged in urine after its administration, suggested that this metabolite was an end product, and not part of the main metabolic route of tryptophan. In these early studies both Ellinger and Homer proposed that a

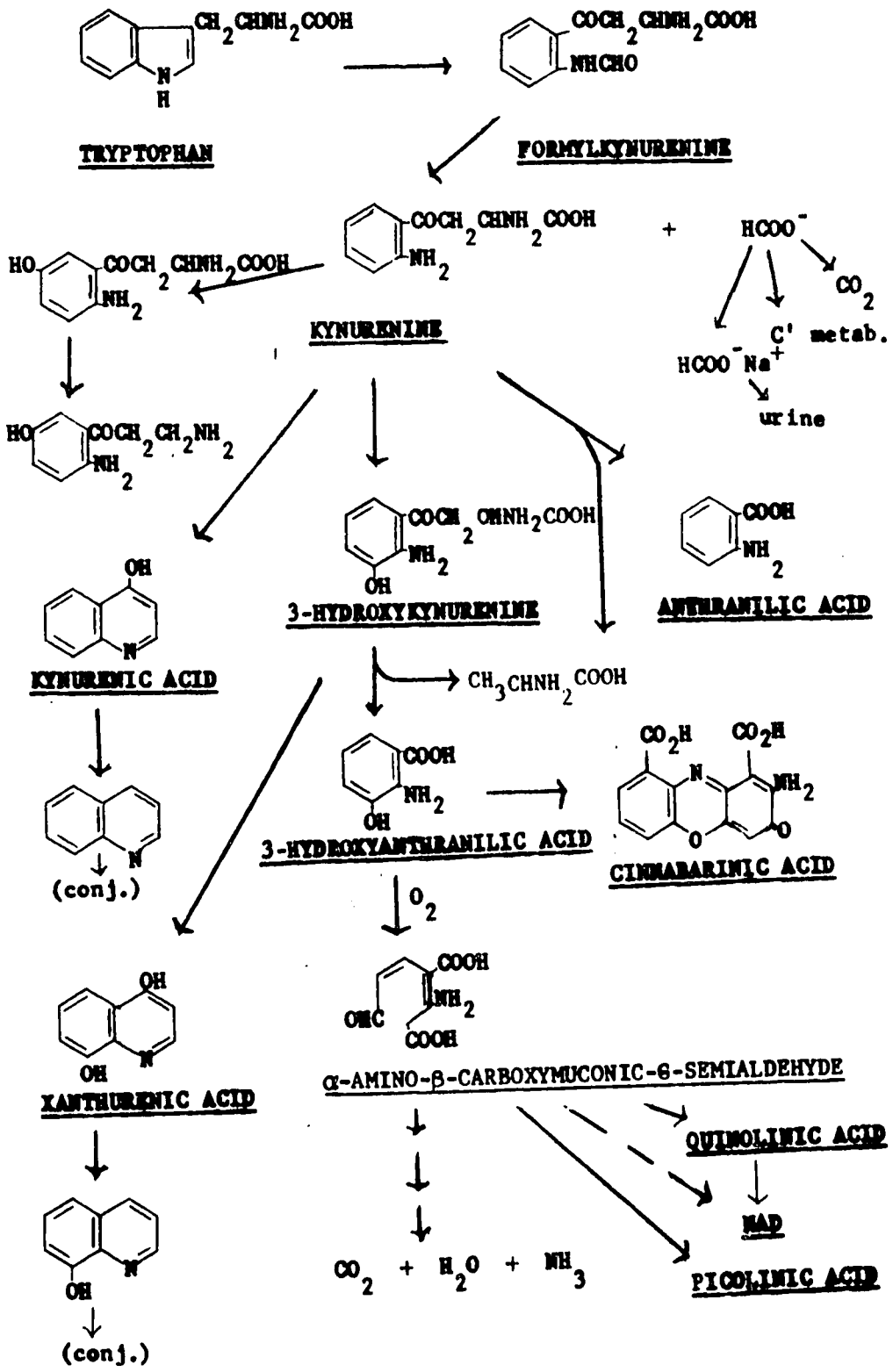


Fig. 2. Catabolism of tryptophan along the pyrrolase pathway

kynurenine-like intermediate had to be formed before recyclization. Kynurenine was found 26 years after the report of Ellinger (56). Other metabolites of tryptophan were discovered in rapid succession. Gradually a metabolic scheme for the degradation of tryptophan and formation of niacin emerged. Kotake and Masayama were the first to describe a system in vitro which converts tryptophan to kynurenine, and they named it tryptophan pyrrolase (57). An account of this enzyme will be given in a separate section. The product of the reaction, formylkynurenine, is acted on by formamidase to yield kynurenine (58). Kynurenine can be metabolized by three major routes: 1) side chain scission to yield anthranilic acid (59), 2) transamination and cyclization to form kynurenic acid (60), 3) ring oxidation to yield 3-hydroxykynurenine (61).

The first reaction is carried out by a B_6-dependent enzyme, kynureninase (61-63) first identified by Braunstein et al.(62). The enzyme is found in the supernatant of liver and kidney (64); it is specific for L-kynurenine or L-3-hydroxykynurenine, and is more active on the latter (65). Kynureninase activity is decreased in B_6-deficiency (62, 63, 65) and kynurenine accumulates (66, 67). Although the enzyme protein remains the same in vitro (65), the measured activity is lower. According to Knox's determinations, the potential activity of tryptophan pyrrolase can exceed that of kynureninase and therefore the latter can become rate-limiting in vivo in B_6-deficiency following induction of tryptophan pyrrolase

(65). That removal of the intact side chain is of physiological importance was shown by Gholson et al. using radioisotopic techniques (68).

Heidelberger et al., employing radioactive tryptophan, demonstrated that kynurenine is converted to kynurenic acid (60) thus confirming earlier reports. Mason and Berg suggested that the reaction was a transamination on the basis of its requirement for pyridoxal phosphate and α-ketoglutarate (63). It is found in rat kidney and liver and occurs both in the supernatant and mitochondrial fractions (69). The kidney enzyme acts on D- as well as L-kynurenine (70). A purified preparation is inhibited by heavy metals, and protected against heat inactivation by both pyridoxal phosphate and α-ketoglutarate (70). The enzyme can use either kynurenine or 3-hydroxykynurenine as substrate but there is some evidence that different enzymes act on these intermediates (71). Thus, Korbitz et al. found that B_6-deficient rats excreted elevated amounts of xanthurenic acid, but not kynurenic acid (67); Porter et al. showed that cortisone had different effects on the urinary excretion of kynurenic and xanthurenic acids in adrenalectomized rats (72). The products of the action of kynurenine transaminase, kynurenic acid and xanthurenic acid, are relatively inert metabolically; a large proportion of the exogenously administered acids are excreted unchanged (73, 74); the rest may be dehydroxylated to quinaldic or 8-hydroxyquinaldic acid (73,

74). Part of this dehydroxylation is attributed to the action of intestinal bacteria (75) as 2/3 of the oral dose of both acids are dehydroxylated but only 2 - 10% of the parenterally administered acids are excreted as quinaldic and 8-hydroxyquinaldic acid. Some kynurenine may be hydroxylated in the five position and then decarboxylated (76). By far the greater part of kynurenine is converted to 3-hydroxykynurenine (77) by a mitochondrial enzyme found in liver and kidney (64). Considering that 89% of L-kynurenine-keto-^{14}C is oxidized to $^{14}CO_2$ (78) and about 72% of the corresponding hydroxy derivative appears as respiratory $^{14}CO_2$ (79), the efficiency of conversion *in vivo* is quite high. Kynurenine hydroxylase requires NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase for activity. Cysteine stimulates and ferrous ions inhibit the enzyme (64).

Two routes are available for the removal of 3-hydroxykynurenine. Transamination and cyclization yields xanthurenic acid, first detected in the urine of vitamin B_6-deficient rats by Lepkovsky *et al.* (80). Using tryptophan labeled with N^{15} Schayer proved conclusively that xanthurenic acid is a metabolite of tryptophan (81) and is the major one in rats as opposed to kynurenic acid (81). However, others have found equivalent (67, 82) or greater amounts of excreted kynurenic acid (67, 83), the latter under conditions of a tryptophan load.

Competing with kynurenine transaminase for 3-hydroxykynurenine is kynureninase which splits the side chain off intact (68) forming 3-hydroxyanthranilic acid. This reaction is far more important than transamination; as mentioned previously, about 72% of this aromatic derivative is attacked by kynureninase, within 12 hours. L-kynurenine is oxidized more rapidly than L-3-hydroxykynurenine (78, 79) *in vivo* although the former is a precursor of the latter. This may be accounted for by the difference in transport of the two compounds, or the possibility that 3-hydroxykynurenine transaminase, if it is a different enzyme than kynurenine transaminase, is more active, or alternate routes for the metabolism of 3-hydroxykynurenine may exist.

At this point it may be pertinent to elaborate further the relationship between these metabolites and vitamin B_6-deficiency. In early studies, kynurenic acid and xanthurenic acid were found in elevated amounts in urine of B6-deficient rats (80, 82). This was interpreted as indicating that the conversion of L-tryptophan to unidentified products was impaired, leading to a shunting of excess metabolites to these quinaldic acids. A theoretical basis for these results came a few years later when Braunstein *et al.* showed that kynureninase which degrades kynurenine was less active in livers of B_6-deficient rats (62). The discovery that 3-hydroxykynurenine was a metabolite of kynurenine, that kynureninase acts on both of these intermediates (61), and that the enzyme

could become rate-limiting in vitamin B_6-deficiency (65) led to a qualitatively satisfactory explanation of the deranged tryptophan metabolism. There are however two discrepancies which have made this explanation less tenable. If both kynureninase and kynurenine transaminase are B_6-dependent enzymes, why is only one affected, and not the other? Ogasawara et al. determined the Km values for both enzymes and found that the Km value for kynureninase is less; therefore theoretically it should function at lower concentrations of substrate than the transaminase (69). However, in B_6-deficiency, whereas the kynureninase and kynurenine transaminase activities are both reduced in the supernatant fraction, the mitochondrial transaminase is fully active (69). This would account for its greater functioning during the deficiency. The second discrepancy is why kynurenic acid excretion in B_6-deficiency is unaffected whereas excretion of xanthurenic acid is very high following a load of tryptophan (67). If transaminase is the same enzyme for both kynurenine and 3-hydroxykynurenine, then xanthurenic acid and kynurenic acid should be excreted in equivalent amounts. The explanation offered by Ogasawara et al. was because both kynureninase and transaminase are lower, anthranilic acid and kynurenic acid formation is impaired leading to a rapid buildup of 3-hydroxykynurenine, which is then transformed to xanthurenic acid (69). Korbitz et al. denied this possibility by showing that kynurenine and 3-hydroxykynurenine

accumulate in equivalent amounts (67). As an alternative, they postulated that 3-hydroxykynurenine transaminase was a different enzyme and less affected by the deficiency than kynurenine transaminase. It should be pointed out that there is no evidence *in vivo*, to the knowledge of the author, that the excretion of anthranilic acid or 3-hydroxyanthranilic acid is reduced in B_6-deficiency without tryptophan loading. In fact, Korbitz *et al.* found anthranilic acid glucuronide and anthranilic acid excretion in equivalent or greater amounts in B_6-deficiency (67). The only other evidence suggesting impairment of kynureninase *in vivo* is the decreased oxidation of tryptophan-7 α-^{14}C in B_6-deficient rats (84). According to the accepted metabolic pathway of tryptophan, $^{14}CO_2$ could only arise in respiratory gases from the benzene ring, following the action of kynureninase.

Two routes of major consequence remain to be outlined: the conversion of tryptophan to niacin and the complete oxidation of this indole to CO_2. A metabolic relationship between tryptophan and niacin was revealed when Krehl *et al.* found that tryptophan could replace niacin in the diet (19). The conversion in mammals of tryptophan $\rightarrow$ nicotinic acid (85) and quinolinic acid (86) and the oxidation of quinolinic acid to N-methylnicotinamide (87) was proved conclusively by radioisotopic experiments. It soon became evident that the transformation of tryptophan to pyridine nucleotides was of physiological importance to some (88, 89)

but not all mammals (90).

The synthesis of NAD from tryptophan, coupled with the finding that tryptophan labeled in the benzene ring is rapidly converted to $^{14}CO_2$ (91) led to an extensive search of the intermediates of this pathway. 3-Hydroxyanthranilic acid was found to be a precursor of quinolinic and nicotinic acid (92, 93, 94). Careful studies by Mehler and May *in vitro* and *in vivo* (94) showed that 3-hydroxyanthranilic acid is split enzymically forming an acyclic compound that either recyclizes spontaneously to quinolinic acid or is acted on by picolinic carboxylase to yield picolinic acid. But there was no evidence for the further oxidation of these quinoline derivatives to CO_2. In the same year that this work was reported, Dalgliesh and Tabechian suggested that this acyclic intermediate may be a precursor of aliphatic compounds which are ultimately oxidized to CO_2 (91). Henderson *et al.* using tryptophan labeled in a specific position on the benzene ring, found a large percentage of the labeled material in respiratory CO_2, thus lending support to this suggestion (95).

It is now generally accepted that tryptophan metabolism diverges into two pathways following oxidation of 3-hydroxyanthranilic acid, a complete degradative pathway (glutarate) and a biosynthetic pathway (NAD pathway).

b. Glutarate Pathway (Fig. 3)

Compelling evidence that 3-hydroxyanthranilic acid is

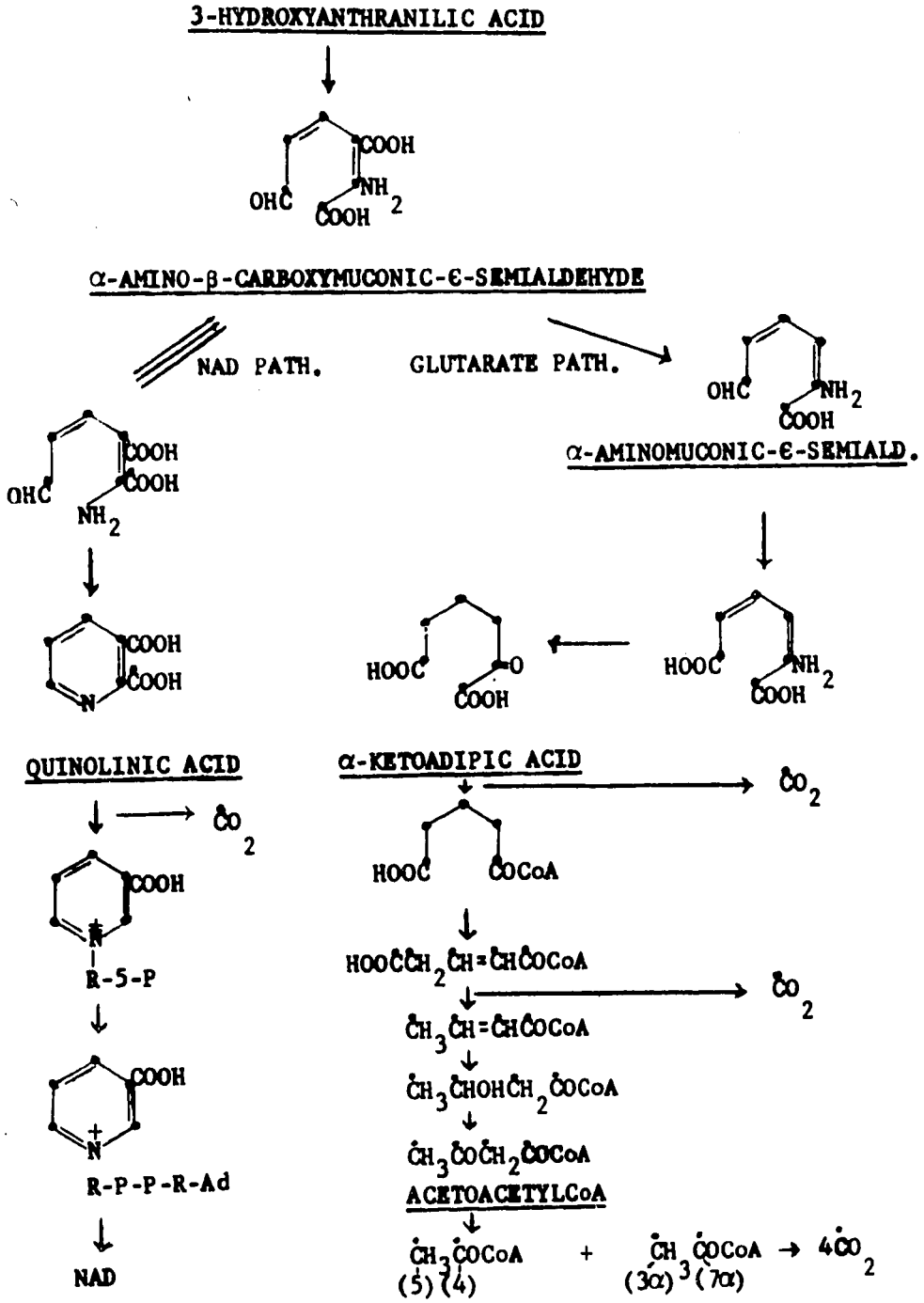


Fig. 3. Catabolism of 3-hydroxyanthranilic acid along glutarate and NAD pathways. Each carbon of the benzene ring is labeled (.) so as to indicate its metabolism.

an intermediate in the total oxidation of tryptophan was presented by Gholson et al. (84) who showed that 3-hydroxyanthranilic acid (3OHAA) labeled with ^{14}C in the benzene ring is rapidly converted to $^{14}CO_2$, and tritium from 3OHAA-^{3}H, appears in urinary glutarate, the suggested intermediate. An enzymic basis for the glutarate pathway has now been described (96 - 99). 3-Hydroxyanthranilic acid oxygenase, found in liver and kidney, converts 3-hydroxyanthranilic acid to an aliphatic compound, α-amino-β-carboxymuconic-ϵ-semialdehyde, by inserting O_2 directly into the ring (100). The enzyme evaded purification until Decker et al., by the judicious choice of factors which maintain the stability of the enzyme, were able to purify it 1800-fold (101). The enzyme activity drops rapidly during the catalytic process, presumably owing to reoxidation of the active ferrous form (102). Other reports have indicated that excess oxygen will inactivate it (103). The catalytic capacity of the enzyme, as measured in vitro, far exceeds its apparent oxidizing ability in vivo (104). The product of the reaction may cyclize spontaneously, leading to the formation of NAD, or may be catalytically oxidized by picolinic carboxylase. This enzyme, found in the supernatant fraction of liver and kidney (105), may be under hormonal control as Mehler et al. found increases in its activity several days after cortisone treatment (106). Its presence in large amounts in the cat has prompted Ikeda et al. to suggest that cats cannot use

tryptophan as a source of NAD because of the high activity of this "diverting" enzyme (105). However Hankes et al. found the conversion of 3-hydroxykynurenine to quinolinic acid equivalent in the rat or cat, implying that the defect is prior to formation of 3-hydroxykynurenine (79). A dehydrogenase, in liver and kidney (98, 104), oxidizes α-amino-muconic-ε-semialdehyde to α-aminomuconic acid in the presence of NAD (98). The enzyme is not specific, oxidizing other aldehydes including formaldehyde, but not as rapidly (98). α-Aminomuconic acid is converted (in the presence of NADH or NADPH) by a reductase to α-ketoadipic (105). The subsequent steps in the oxidation are outlined in Fig. 3.

c. NAD Pathway (Fig. 3)

In 1963 Nishizuka and Hayaishi presented evidence that 3-hydroxyanthranilic acid is converted to niacin ribonucleotide in vitro (107, 108). Until then, the conversion was inferred from in vivo studies, because no further metabolism of quinolinic or picolinic acid could be detected in tissue preparations (94). A soluble enzyme found in kidney and liver, quinolinate transphosphoribosylase, transfers 5-phosphoribosyl-1-pyrophosphate (P-R-PP) to quinolinate (108). This rate-limiting step can occur only in kidney and liver in the presence of a divalent metal ion and P-R-PP (109). Nishizuka and Hayaishi postulated a quinolinic acid mononucleotide intermediate (108), but Gholson et al. could find no evidence for it (109). That quinolinic acid is the inter-

mediate in the biosynthesis of NAD was strongly supported by the demonstration that this acid is a better substrate for transphosphoribosylase than nicotinic acid and has a lower Km value (109). Gholson et al. pointed out that the poor absorption of quinolinic acid probably accounts for the fact that this acid is not an adequate dietary substitute for niacin (109). More recently, however, Hankes et al. questioned the likelihood of a physiological conversion of quinolinic acid to pyridine nucleotides (79). They based these doubts on the finding that the D- and L-isomers of 3-hydroxykynurenine-^{14}C were converted to about the same extent into nicotinic acid, but the L- was a far better precursor of quinolinic acid.

In addition to being oxidized along the NAD and glutarate pathways, 3-hydroxyanthranilic acid is also converted to cinnabarinic acid (110, 111). This transformation was suggested by Morgan et al. as an alternate route for the metabolism of the intermediate in poikilothermic vertebrates (110) but this has been challenged (104).

While the pathway for the total catabolism of tryptophan -- as outlined above -- appears to be the most plausible at present, there is some evidence which casts doubt upon it.

Thus Rothstein and Greenberg demonstrated that a preparation of rat liver was able to oxidize tryptophan-7α-$^{14}C \rightarrow {}^{14}CO_2$ at a faster rate than kynurenine, labeled in the equivalent position (112). Alteration of the medium

resulted in a reversal of the rates of oxidation of the two compounds (112).

Other evidence that alternate pathways exist for the total catabolism of tryptophan was presented by Lan and Gholson: Both the snail and earthworm readily oxidize tryptophan-5-$^{14}C \longrightarrow {}^{14}CO_2$, yet none of three important enzymes of the glutarate pathway could be detected in these invertebrates (104).

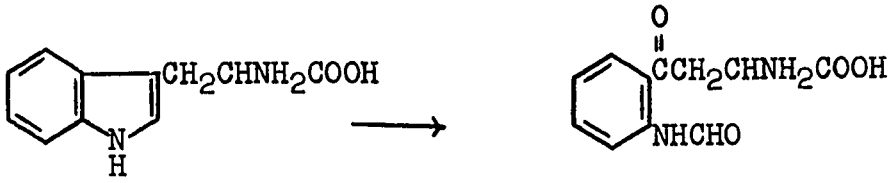
Confirmation of a different catabolic route for tryptophan awaits isolation of the intermediates.

3. Tryptophan Pyrrolase

A liver preparation, able to cleave the pyrrole ring, was first reported by Kotake and Nasayama, and termed tryptophan pyrrolase (57). But it was not until Knox and Mehler completed a careful study of the system (113) that investigations of the enzyme entered into a phase of intensive inquiry. As a result of their work the enzyme became a challenge for researchers in two major spheres of biochemistry: enzyme mechanisms and enzyme induction.

a. Mechanism of Action

Tryptophan pyrrolase [L-tryptophan: H_2O_2 oxidoreductase E. C. 1.11.1.4] catalyzes the following reaction:



The enzyme is specific for L-tryptophan, although Hayaishi claims to have isolated a pyrrolase from intestine which is specific for D-tryptophan (127).

In their first paper, Knox and Mehler suggested that the enzyme was a coupled peroxidase-oxidase, because the overall reaction was inhibited by catalase and the addition of an accessory system for the generation of H_2O_2 reversed the inhibition (113). This reaction mechanism was accepted until Hayaishi *et al.* showed that molecular O_2^{18} was inserted directly into the indole ring (114); Tanaka and Knox further supported the view of direct oxidation by showing that the activity was dependent on the O_2 content of the reaction vessel, and that H_2O_2 was needed only at the onset of the reaction (115). (More recently Knox and Ogata have demonstrated a requirement for a reducing agent for the duration of the reaction (116).

The enzyme was isolated from *Pseudomonas* in sufficiently pure form to establish its hemeprotein nature (115, 117). The iron porphyrin was identified as hematin (118) and the activating effect of microsomes (118-120) was attributed in part to the release of hematin from this particulate fraction. More recent reports suggest that methemoglobin is a better external source of cofactor than hematin (121, 122). Subsequent purification of the mammalian enzyme showed that it had an absolute requirement for hematin (123). The hematin (or methemoglobin) is considered a cofactor rather than

a prosthetic group because it readily dissociates from the enzyme (123). Binding of the substrate and cofactor is facilitated by the presence of either as the Michaelis constant for one decreases in the presence of the other (123).

There are presently three different hypotheses regarding the valence state of the iron during catalysis:

1) following binding of the oxidized iron porphyrin to the enzyme the iron is reduced at the onset of the reaction (by an external reductant) and the divalent active form persists unchanged during the catalytic process (115, 116).

Thus: $E \xrightarrow{+\text{Hematin (H)}} EH^{+3} \xrightarrow{+\text{Reduction}} EH^{+2}$ (active)

This explanation accounted for the observation that the reaction is activated by reducing agents such as ascorbate, H_2O_2, or a peroxide-generating system (113, 115, 116), if added after hematin. Knox and Ogata have suggested that the reducing agent accelerates the initial conversion of the inactive ferric form into the active ferrous form and is necessary throughout the reaction to maintain the iron in a reduced state (116). Endogenous reducing agents would account for the activation that occurs in some hematin-treated preparations to which a reducing agent has not been added (124).

2) Hematin is active in the trivalent form but may undergo oscillations in valence state in a manner analogous to cytochrome c (125, 126). Feigelson et al. showed that during anaerobic incubation of the enzyme, hematin, and substrate, a 552 mμ band appears, characteristic of a reduced

porphyrin. Admission of oxygen into the reaction vessel eliminates this band. Thus they proposed this scheme:

$$TRY + HFe^{3+} \longrightarrow HFe^{2+} + TRY^{ox}$$

$$O_2 + HFe^{2+} \longrightarrow HFe^{3+} + O_2^{red.}$$

$$TRY^{ox} + O_2^{red} \longrightarrow FK$$

3) The divalent form is the active form but it may undergo cyclic oxidation and reduction. Hayaishi proposed that the divalent iron, after forming a ternary complex with tryptophan and oxygen, may be replaced by other metals such as copper (127). Recently the participation of enzyme-bound copper in the microbial tryptophan pyrrolase reaction was suggested by the presence of copper in the purified enzyme and the sensitivity of the catalytic process to copper chelating agents (128). It is yet to be shown that copper does participate in the reaction in the manner proposed by Hayaishi.

b. Properties of Tryptophan Pyrrolase in vitro

Several different forms of the enzyme exist in vitro: 1) endogenous holoenzyme, which is the active form of the enzyme, readily assayed in unpurified preparations; 2) endogenous apoenzyme which can be activated by addition of hematin (118) or methemoglobin (121, 122) or a source of hematin; 3) an inactive oxidized holoenzyme (129) which can be reactivated readily by preincubation with substrate and a reducing agent (129); and 4) latent forms (121, 122, 130, 131) which may be conjugated or unconjugated, and can be recognized after a) preincubating a concentrated preparation of liver with

tryptophan (aerobically or anaerobically) (122); b) preincubating a dilute preparation with a heat sensitive particulate fraction (131); or c) incubating the enzyme alone anaerobically (130).

Other activators of the enzyme include boiled liver cell sap (132), cyclic-3',5'-AMP (cAMP) (122, 132-134), other purine derivatives (133, 134), RNA (135), $HgCl_2$ or p-choromercuribenzoate (136) and microsomal lipids (137). The relationship between the various forms of the enzyme and these activators remains obscure. The procedure used to prepare the enzyme most likely plays a very important role in determining its final activity (122, 131, 138).

In cell-free preparations the enzyme is very unstable without added substrate, but can be protected against inactivation by its substrate and other aromatic compounds (113, 139-141). The inactivation is not a consequence of proteolysis (which has been shown to occur in liver slices or <u>in vivo</u>) as the enzyme protein remains fully active immunologically (130). Knox and Piras claim that the enzyme becomes inactivated because the active reduced form of the Fe^{++} is oxidized during preincubation; agents which protect the enzyme against inactivation do so by preventing the oxidation of the Fe^{++}. The process is reversible as the enzyme can be reactivated by preincubation with tryptophan and a reducing agent (129).

Several inhibitors of tryptophan pyrrolase have been

identified in vitro including adrenaline, serotonin (143), a host of tryptophan analogues (141, 143), other aromatic substances (144) and steroids (145). These will be dealt with in a separate section.

c. Regulation of Tryptophan Pyrrolase Activity

The activity of tryptophan pyrrolase varies widely under different physiological or pathological conditions.

The enzyme is absent in the fetus, and at birth, but can be detected within the first day (146) to two weeks after birth (147). The mechanism which triggers the appearance of the enzyme remains obscure, but following its initial appearance, the enzyme activity rises gradually until it reaches adult levels.

Another factor which may regulate the amount of active enzyme is the availability of cofactor. Thus administration of hematin alone causes saturation of the apoenzyme thereby increasing the amount of fully active enzyme. This process of saturation does not, however, stimulate the formation of new apoenzyme (148). The initial rise in tryptophan pyrrolase following treatment with tryptophan is a consequence of saturation of the enzyme with cofactor (141). In experimental porphyria the increased availability of endogenous iron protoporphyrin is said to account for the high levels of pyrrolase (149). Other proposed regulators of the enzyme include insulin (150), the hypothalamus (151, 152) and cyclic-3', 5'-AMP (132). Chytil and Skrivanova suggested that cyclic-3',

5'-AMP may activate tryptophan pyrrolase in a manner analogous to its effect on phosphorylase. They based this hypothesis on the finding that cyclic-3', 5'-AMP reactivates inactivated tryptophan pyrrolase in vitro (132). The theory has been weakened by two independent observations: Many purines or purine derivatives have been identified with the same or greater capacity in vitro (133, 134); Knox, Piras and Tokuyama showed that cyclic-3', 5'-AMP will substitute for ascorbic acid as a reductant of the inactive ferric form of the enzyme. Thus its action as an activator may be quite non-specific, (122).

Other agents, which damage the liver, such as carcinogens (153), CCl_4 (154), x-irradiation (155), or hepatomas (153, 156) render it unable to maintain normal levels of tryptophan pyrrolase or to respond normally to regulators of the enzyme.

Two factors are the important physiological regulators of tryptophan pyrrolase: its substrate (10) and the adrenal-pituitary system (or an external source of corticoids) (21, 157). Following administration of substrate or hydrocortisone a rise in the activity of the enzyme can be detected within 2 hours. Elevated levels persist for several hours, but at the end of a 24 hour period the activity is restored to normal.

A number of reports have indicated that either type of induction is accompanied, in part, by <u>de novo</u> protein synthesis,

rather than by a general proliferation of liver cells, or by activation of preformed enzyme. The fact that the induction occurs within 6 hours of presentation of the inducing agents and is over within 18 hours precludes a growth of new cells (21). The induction by both agents is prevented by inhibitors of protein synthesis (158-162). The strongest evidence that the induction is accounted for by new protein was presented by Feigelson and Greengard in 1962: they showed that administration of tryptophan or cortisone causes an increase in the enzyme activity and an equivalent rise in the concentration of a protein immunochemically indistinguishable from tryptophan pyrrolase (163). This has been confirmed recently (142).

Although both substrate and hormone result in augmented enzyme protein levels, it has become increasingly obvious that the inducing agents act through different mechanisms. Civen and Knox ruled out the possibility that cortisone, because of its general catabolic effect, increases the levels of tryptophan in the liver, thereby acting through tryptophan (22). Cortisone induces the enzyme in isolated perfused liver, indicating that it acts directly on the liver cells (162). The inductions by tryptophan and cortisone are additive, implying separate mechanisms (122). (Schimke et al., on the other hand, found a synergistic effect (142) but Knox et al. pointed out that their assay method probably did not reflect accurately the activity of pyrrolase (122).) In addition,

tryptophan could evoke a rise in the enzyme activity in the absence of the adrenal glands, suggesting that it was not acting through adrenocortical hormones (157).

1. Substrate induction

The induction of tryptophan pyrrolase by substrate can be accounted for by several mechanisms, including de novo protein synthesis, activation of preformed enzyme by cofactor or other means, and enzyme stabilization. The mechanism that has recently received the most acceptance is the stabilization-hypothesis. Accordingly, tryptophan causes a buildup of the hepatic enzyme levels by preventing degradation of newly-synthesized enzyme, which otherwise is being constantly formed and destroyed. A brief history of the development of the stabilization hypothesis is offered.

In 1950-51 tryptophan was shown to induce the enzyme and protect it against spontaneous inactivation in vitro (10, 113). Later Sourkes and Townsend showed that α-methyltryptophan is capable of both actions (139). The connection between enzyme induction and enzyme stabilization gave credence to the view of Dubnoff and Dimick (164), that substrate induction is a process of enzyme stabilization in vivo. This view was temporarily weakened by the discovery that many tryptophan analogues can stabilize the enzyme in vitro, yet not induce it in vivo (140, 141).

More recently Schimke, Sweeney and Berlin have reinstated the stabilization-hypothesis with convincing evidence: the time

course of the increase in enzyme activity produced by hydrocortisone or tryptophan or both fitted a theoretical model in which cortisone stimulated synthesis of the enzyme and tryptophan delayed enzyme degradation; the effects of both were synergistic. Furthermore an increased incorporation of radioactive leucine was demonstrable only in the enzyme induced by cortisone, implying accelerated protein synthesis. Tryptophan did not stimulate incorporation of radioactivity into protein but did prevent the loss of radioactivity from preformed enzyme (142). This result suggested that tryptophan protects the enzyme against degradation by proteolysis.

Further evidence stemmed from in vitro studies. Tryptophan and α-methyltryptophan were shown to possess unique properties with respect to other tryptophan analogues. Thus, although many analogues stabilized the enzyme against heat inactivation, only tryptophan and α-methyltryptophan were able to prevent proteolysis of the enzyme in vitro (130).

Hence the ability of a substrate-like compound to induce the enzyme would be governed by whether it can protect it from proteolysis. This hypothesis can readily be accommodated by previous information about the enzyme: the enzyme has a rapid turnover (159), a property that would be essential in order that a steep rise in activity occur within a short time, by a stabilization mechanism. Inhibitors of protein synthesis would still prevent the induction because

de novo synthesis but not accelerated synthesis is necessary for this type of mechanism.

Another mechanism proposed to account for the substrate induction is the formation or release of enzyme protein, stimulated by an increased production of holoenzyme from apoenzyme. Feigelson et al. found a greater degree of saturation of the enzyme with cofactor within 2 hours of treatment with tryptophan. This was followed by a rise in total activity, the holoenzyme to apoenzyme ratio remaining high (141, 165). That tryptophan may act by influencing the binding of cofactor with the enzyme was borne out by studies in vitro: Thus, these authors showed that tryptophan does facilitate the formation of the enzyme-cofactor complex (123). Furthermore it can affect the partitioning of hematin between the enzyme and other heme-binding proteins. These findings led them to suggest that a more rapid formation of enzyme-cofactor complex, effectuated by tryptophan, would stimulate a further synthesis or release of apoenzyme from the template site by mass action or other means. The hypothesis was further advanced by Greengard, who found that only tryptophan and α-methyltryptophan, of several tryptophan analogues studied, can induce the enzyme and shift the holoenzyme : apoenzyme ratio (166, 167). In all probability, the ultimate regulator will prove to be a composite of enzyme stabilization, activation, and de novo synthesis.

ii. Hormonal induction

Many substances are able to induce tryptophan pyrrolase in intact animals, but become ineffective if the adrenal glands are removed (157). Only corticosteroids (other than substrate-like compounds) are capable of augmenting the enzyme levels in adrenalectomized rats. This discovery led Knox aud Auerbach to surmise that the enzyme is under the control of two factors, and all other compounds which raise the levels of the enzyme in intact animals, dc so by a stress-mechanism (157).

The mechanism by which adrenocorticosteroids increase the activity of tryptophan pyrrolase is being slowly elucidated. The induction requires DNA-dependent RNA synthesis (161) and gives rise to new protein (142, 163), but there is no shift in the holo : apoenzyme ratio (166). Much of the newly-synthesized enzyme, as measured in vitro, remains in the latent form (122, 131). A more rapid turnover of RNA (141, 170) and a decline in nuclear histones (171) accompany the induction.

It is interesting to speculate on the physiological roles of the inductions by substrate and hormone. Moran and Sourkes showed that the induction of tryptophan pyrrolase did cause an increased destruction of exogenous tryptophan (168) and suggested that physiological significance be attached to the process. It is relevant to inquire whether the products of the pyrrolase pathway possess physiological

activity so as to extend the role of the induction of the enzyme. Increased tryptophan pyrrolase leads to the formation of oxidized pyridine nucleotides, which may serve an important function during stress (154). Berry and Smythe have provided evidence that metabolites along the pyrrolase pathway protect mice against bacterial endotoxin, implying that the bacteriostatic effect of cortisone is related to its ability to induce tryptophan pyrrolase (172).

Feigelson and Feigelson have suggested that the increased glutamate, formed from tryptophan and tyrosine following induction of tryptophan pyrrolase and tyrosine transaminase by cortisone, is the mediator of the catabolic effect of corticoids in lymphoid tissue (173).

The formation of these metabolites during a stress response may very well suggest a specific role for the induction of tryptophan pyrrolase by cortisone. On the other hand, Berlin and Schimke state that cortisone has a general anabolic effect on the liver, thereby increasing the activity of several enzymes. Its action on pyrrolase appears specific only because of the sharp rise in activity of the enzyme, a consequence of its rapid turnover rate (174).

D. Effect of Drugs on Tryptophan Metabolism

Drugs have been used extensively in studies of the biochemistry of tryptophan in higher organisms. The majority of such studies can be classified readily into two quite distinct lines of investigation, having different objectives. On the one hand, there has been the investigation of the 5-hydroxylative pathway the main purpose of which is to obtain more information about the biologically active compound, serotonin. On the other hand, there has been investigation of the main catabolic pathway with the objective of elucidating the mechanism of action, and of induction, of the enzyme tryptophan pyrrolase. In this section, a brief review of the literature concerning this topic is given.

1. 5-Hydroxylative Pathway

Initially, drug research was concerned with inhibition of the pharmacological actions of serotonin. Many compounds were discovered which antagonized the action of serotonin on smooth muscle including serotonin analogues, e.g. N, N-dimethyltryptamine (175), and indole alkaloids such as yohimbine (176), ergotamine (175, 176), dihydroergotamine (175), L.S.D. (175) and harman derivatives (176). The tryptamine backbone of these indole alkaloids prompted Shaw and Woolley to suggest that they are antimetabolites of serotonin (176).

It was of interest to determine whether some indole

alkaloids are antimetabolites of tryptophan as well. A few of the indole alkaloids studied are illustrated in Fig. 4. Later, as the metabolic pathway for the formation and degradation of serotonin was elucidated (20), compounds were sought which would inhibit individual enzyme reactions along this pathway.

Tryptophan hydroxylase, the enzyme catalyzing the initial step in the formation of serotonin, is inhibited by catechols in vitro (38, 177) and in vivo (177). D-Tryptophan is a potent inhibitor of the enzyme extracted from brain tissue (38). The decarboxylation of 5-hydroxytryptophan is inhibited by several drugs; of relevance to this report are the α-methyl derivatives of tryptophan including α-methyltryptophan (178), α-methyl-5-hydroxytryptophan (178, 179) and α-methyltryptamine (180). Monoamine oxidase which degrades serotonin can be antagonized by many amine derivatives; among these are α-ethyl- and α-methyltryptamine (180) and N,N-diethyltryptamine (181), all three of which inhibit the enzyme in vivo and in vitro.

2. Pyrrolase Pathway

Drug studies of this pathway have been chiefly concerned with induction and inhibition of tryptophan pyrrolase.

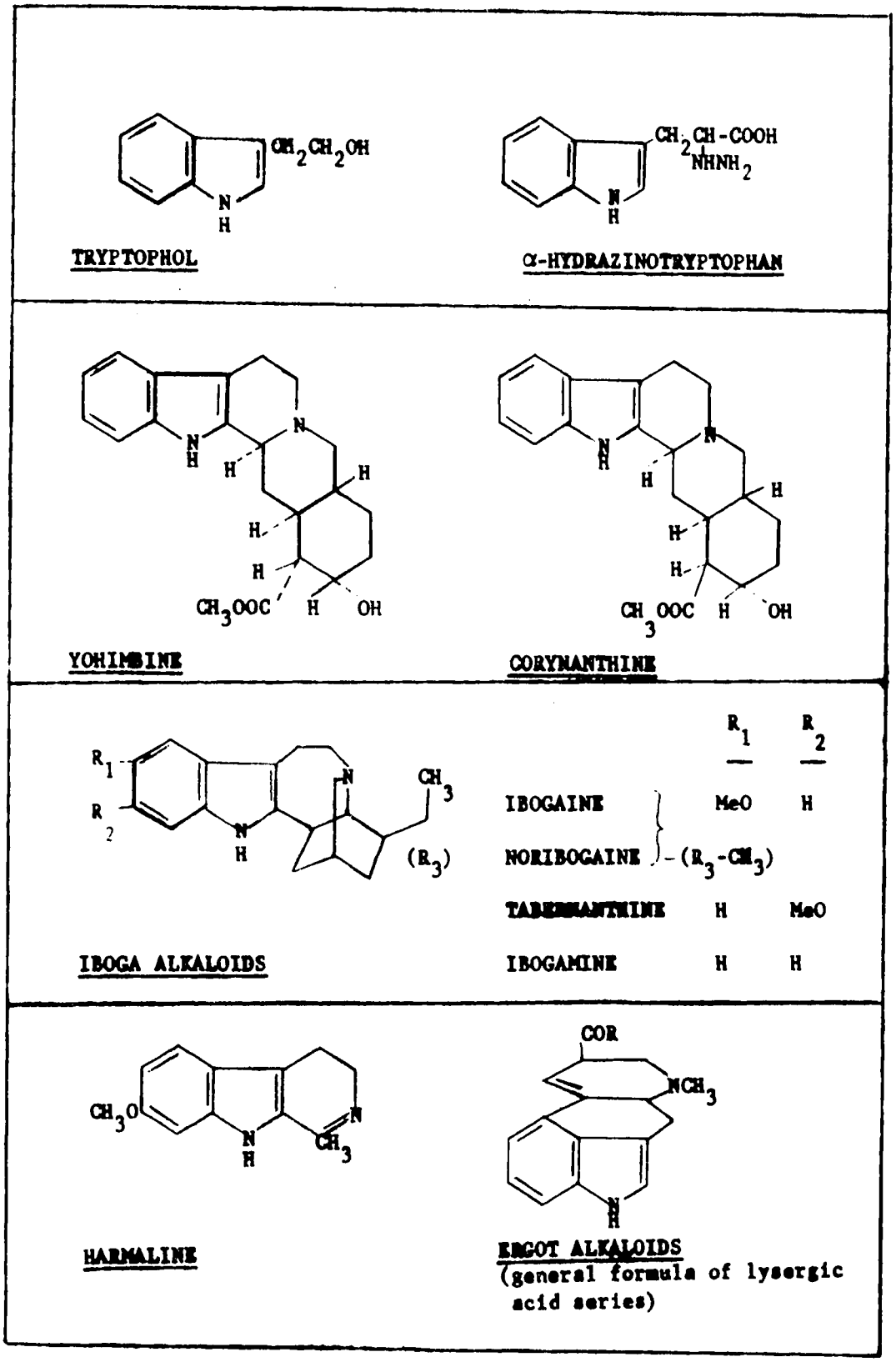


Fig. 4. Structure of several indoles studied

a. Induction by Tryptophan Analogues.

Many drugs cause an adaptive increase in the activity of this enzyme in intact animals, by a stress mechanism. A survey of these compounds is beyond the scope of this review. Thus far, only three substances are known which increase the activity of tryptophan pyrrolase *in vivo*, in a manner analogous to tryptophan; these are D-tryptophan, N-acetyltryptophan and α-methyltryptophan (139, 140). The induction by the first two substances may result from their conversion to tryptophan itself, by transamination and hydrolysis, resp. It is unlikely that α-methyltryptophan is demethylated to yield tryptophan.

Although the α-methyl analogue possesses many properties in common with tryptophan, there are a few differences in their action on pyrrolase: (a) Both increase the levels of the enzyme within 6 hours (139, 140) but the induction by tryptophan is over within 24 hours (21) whereas the effect of α-methyltryptophan persists as long as one week (168, 169). (b) Moreover maximal levels of the enzyme are attained more slowly if α-methyltryptophan is used as the inducing agent (168). The delayed rise in tryptophan pyrrolase may be related to the slight inhibitory effect of the amino acid analogue (130, 139) on the enzyme. (c) α-Methyltryptophan causes a weight-loss in rats, concomitant with the induction. Sankoff and Sourkes have suggested that the weight loss is a consequence of a mild tryptophan deficiency brought about by the

increased destruction of tryptophan during the prolonged induction period (169).

b. Inhibitors of Tryptophan Pyrrolase

One of the first compounds to be recognized as an inhibitor of tryptophan pyrrolase in vitro was α-methyltryptophan (139), a weak, competitive inhibitor (KI: 1×10^{-2}M) (130). Another property of the compound is its ability to stabilize the enzyme against inactivation by heat, urea and proteolysis by trypsin (130). It is also capable of activating the enzyme under certain conditions (130) but far less effectively than tryptophan (122).

A year following the initial report on the α-methyl analogue, Ichihara et al. observed that 5-hydroxytryptophan reduced the formation of kynurenine from tryptophan by 89%, if both indoles were present at equimolar concentrations (182). In a study of tryptophan analogues as inducers, stabilizers and inhibitors of the enzyme, Civen and Knox reported that both DL-tryptazan and 5-methyl-DL-tryptophan lower the activity of tryptophan pyrrolase in vitro, (140).

A very extensive study of inhibitors of tryptophan pyrrolase was carried out by Frieden, Westmark and Schor (143). They indicated that many tryptophan analogues effectively block the conversion of tryptophan to kynurenine. Side-chain variants such as tryptamine and indole-3-acetic acid were

competitive inhibitors of the enzyme whereas ring-substituted compounds inhibited the enzyme non-competitively. Serotonin and 5-hydroxytryptophan were the most potent of the latter group. The pyrrole ring was not a structural requirement for inhibition. Thus, many phenols, including adrenaline and noradrenaline lowered the activity of the enzyme. The possibility that phenols act at the proposed binding site of the Fe cofactor was raised. The authors also raised the question of the possible physiological significance of the inhibition by serotonin and adrenaline in vitro.

More recently Wagner has reported on other non-indolic substances which reduce the activity of the enzyme (144). Of significance is that these compounds are metabolites of the pyrrolase pathway. At a concentration of 5×10^{-4}M, 3-hydroxyanthranilic acid, kynurenine, desamido-NAD, nicotinic acid mononucleotide reduce the activity of the enzyme, in decreasing order of effectiveness. The inhibition by 3-hydroxyanthranilic acid and NAD was competitive. The implications of these findings is that intermediates may exert feed-back control on tryptophan pyrrolase in vivo.

Another group of compounds, structurally unrelated to tryptophan, which inhibit the enzyme in vitro, are estrogens. Oelkers and Nolten showed that estrone sulfate and other free estrogens reduced the activity of the enzyme. The inhibition developed depends on the time of addition of the inhibitor to

the incubation medium. Because the inhibition depended on the order of addition of constituents, they postulated competition of estrogen and hematin for the apoenzyme (145).

Many inhibitors of the enzyme stabilize it against spontaneous heat inactivation, including 5-methyltryptophan (130, 140), tryptazan (140), tryptamine (141), indole-3-acetic acid (141), indole (130). This is to be expected because both processes imply an affinity of the compound for binding site(s) on the enzyme.

3. Adrenergic Blocking Agents

It has been indicated that tryptophan does have pronounced physiological actions (Section I, B). Yet the catabolism of tryptophan has not been adequately studied with the aid of drugs as was done with serotonin. The effect of several tryptophan analogues and indole alkaloids (Fig. 4) on the rate of oxidation of tryptophan *in vivo* was studied. In the course of these studies it was noted that yohimbine reduced the rate of metabolism of tryptophan. One of the known pharmacological actions of yohimbine is its adrenergic blocking effect. It was therefore considered of interest to determine whether other adrenergic blocking agents exert this effect on tryptophan catabolism. Both α - and β -adrenergic blocking agents (Fig. 5) were used.

These drugs oppose the physiological and metabolic res-

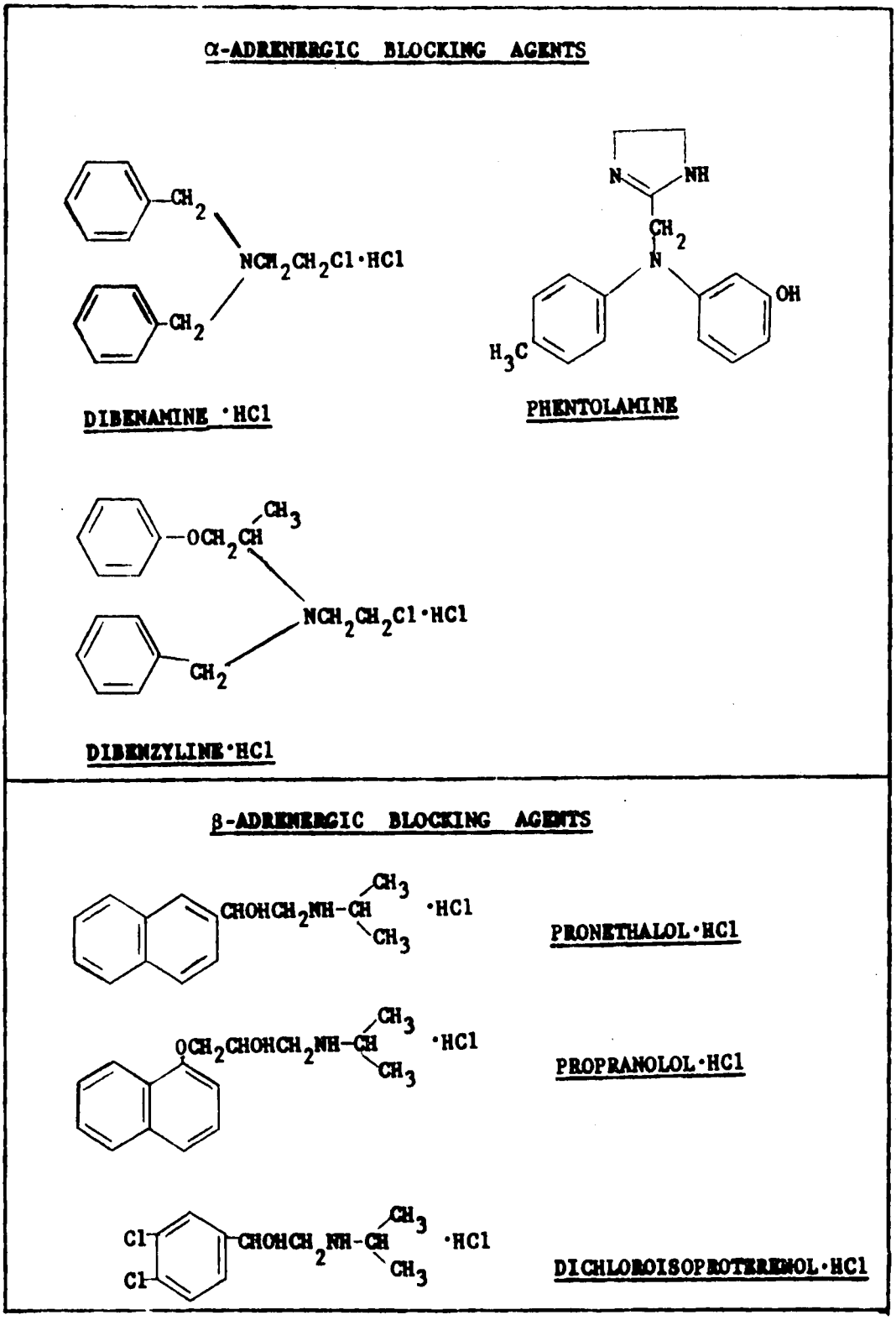


Fig. 5. α - and β -Adrenergic blocking agents

ponses of tissues to adrenergic agents. Their site of action is at the hypothetical adrenergic receptors, which are classified as either α - or β -receptors, on the basis of the receptor hypothesis advanced by Ahlquist in 1948 (183). Before that time two types of receptor were believed to exist, one concerned with inhibitory responses, the other with excitatory responses to adrenergic agents. Ahlquist acknowledged the existence of two types of receptor but proposed that each has mixed functions, the individual function depending on the organ in which the receptor was located.

More recently the receptor hypothesis has been expanded to include metabolic receptors; these are concerned with the lipolytic and gluconeogenic responses to adrenaline. This receptor may be identical with adenyl cyclase, the enzyme which synthesizes cyclic-AMP. The latter compound is believed to be a "messenger" in the series of events which entail the metabolic -- and perhaps -- some physiological response to adrenergic agents (184).

a. α-Adrenergic Blocking Agents (Fig. 4, 5)

Historically, the α-adrenergic blocking agents play a very important role in the development of the α-, β -receptor hypothesis. The classic studies of Dale in 1906 demonstrated that ergot can reverse many, but not all, responses to circulating adrenaline (185). Later, other natural (yohimbine,

corynanthine) and synthetic (dihydroergotamine, dibenamine, dibenzyline) drugs were shown to block several excitatory responses to adrenaline, but not the cardiac response, in mammals (186). These findings encouraged the classification of the drugs as blocking agents, specific for one type of receptor.

The ergot alkaloids have a low degree of specificity as blocking agents (186). The polypeptide part of the ergot derivative is believed to render them blocking agents (186); hydrogenation of ergotamine increases its potency as an antagonist. Both dihydroergotamine and ergotamine block the metabolic response of liver to adrenaline (188).

Yohimbine, another indole alkaloid, has a very specific, yet short duration of blocking effect (186). Its geometric isomer, corynanthine, exhibits similar properties but is more active than yohimbine. Both of these alkaloids exert many actions other than adrenergic blockade; this subject has been reviewed by Nickerson (186).

Of the synthetic compounds, dibenzyline and dibenamine have been used most extensively in the study of adrenergic blockade. The specificity of these β-haloalkylamines is very high and the duration of action is long (186). Both compounds are very reactive chemically. The β-chlorine is readily lost in neutral or alkaline solutions with formation of an ethylenimmonium ring (187). This may react with water or with a

variety of chemical groups including sulfhydryl, amino and carboxyl groups of free amino acids or of protein-bound amino acids (189). The high reactivity of these compounds provides a theoretical basis for their irreversible blocking effect.

Axelrod, Aronow and Brodie studied the distribution of dibenamine (190) and dibenzyline (191). Within two hours of administration, the concentration of these drugs in liver declined to levels too low to measure, but within 4 hours very high levels were detected in adipose tissue. They postulated that the so-called irreversibility of the blocking effect was not related to the chemical binding of the drug with the receptor, but rather a result of its slow release from fat depots. Horowitz and Nickerson, using dibenzyline-^{14}C could detect no accumulation of radioactivity in fat depots (192) and reemphasized the irreversible nature of the receptor-drug complex.

b. β-Adrenergic Blocking Agents (Fig. 5)

The specific type of blocking action by the α-blocking agents strongly supported the view that a single receptor was responsible for most excitatory and some inhibitory responses. In 1958 Powell and Slater reported that dichloroisoproterenol (DCI) blocked most of the adrenotropic inhibitory responses and the cardiac excitatory response (193). This finding was considered convincing evidence for the β-receptor, as proposed by Ahlquist.

Several other β -adrenergic blocking drugs have been discovered since the report of Powell and Slater, including pronethalol and propranolol. Both DCI and pronethalol block the metabolic responses to adrenaline as well as the physiological (188). Phosphorylase stimulation is inhibited partially by DCI and pronethalol in liver, and completely in muscle (188).

In addition to their adrenergic blocking action, some β-blocking agents exhibit other properties; both propranolol and pronethalol are CNS depressants (194) and inhibitors of monoamine oxidase (195). Pronethalol antagonizes the cardiovascular effects of α-adrenergic blocking drugs, including dibenzyline, phentolamine and dihydroergotamine (196). Propranolol, and others, cause an increase in plasma free fatty acids, as does adrenaline, within 60 minutes of administration, an effect which is prevented by pretreatment with dibenzyline (197). The antagonism between α - and β -blocking agents, with respect to lipomobilization and cardiovascular responses, may indicate that the β -blocking agents are partial or weak antagonists of adrenaline.

The pharmacological half-life of propranolol and pronethalol is 35-60 minutes. The metabolic half-life is equally rapid (41-65 minutes) (198). Following administration, very little of both drugs can be detected in liver, with respect to other organs (198).

Few studies on the effects of the blocking agents on

tryptophan metabolism are reported. It is known that both ergotamine and dibenamine block the serotonin-induced stimulation of rat uterus (175). With respect to the catabolic pathway of tryptophan, Maffei-Faccioli showed that the induction of tryptophan pyrrolase by reserpine was not inhibited by dibenamine, ergotamine or phentolamine (199).

II. EXPERIMENTAL

A. Materials

1. Radioactive Materials

Crystalline DL-tryptophan-3'-^{14}C (Sp. act. 4.08-10.3 mc/mM), L-alanine-u-^{14}C in a 0.5 ml solution containing 0.01N HCl (Sp. act. 111 mc/mM) were obtained from New England Nuclear Corp.,(Boston, Mass). DL-Tryptophan-2-^{14}C (pyrrole ring), (Sp. act. 1.08 mc/mM) was purchased from Nuclear Res. Chem. (Orlando, Fla.). Nuclear-Chicago supplied the DL-tryptophan-benzene-(uniformly-labeled)-^{14}C (Sp. act. 3.81 mc/mM), and Calbiochem. the DL-tryptophan-^{14}COOH (10 mc/mM). L-Tryptophan-^{14}COOH (Sp. act. 8.3 mc/mM) was a gift of Schering Corp. Na formate (13 mc/mM) was acquired from Merck, Sharp and Dohme.

Tritiated DL-α-methyltryptophan.H_2O was obtained by exposing one gram of this amino acid to tritium gas by the Wilzbach technique. The purification procedure is described under "Methods". We are indebted to Dr. S.G. Mason and Dr. I. Wadhera of the Pulp and Paper Research Institute of Canada (Montreal) for carrying out the tritiation.

All radioactive compounds were dissolved in a small volume of water and kept frozen at -20° C until use. On the day of an experiment an aliquot of the stock solution was diluted and unlabeled material was added, if necessary, according to the requirements of the experiment. From time to time, samples of the stock solutions of tryptophan were

checked for purity.

2. Drugs and Other Chemicals

DL-α-Methyltryptophan.H_2O, DL-α-hydrazinotryptophan, α-hydrazino-5-hydroxytryptophan, α-methyl-5-hydroxytryptophan, and the tyrosine analogues were generously donated by Merck, Sharp and Dohme (Rahway, New Jersey). The ergot alkaloids (lysergic acid diethylamide, ergotamine tartrate, dihydroergotamine methanesulfonate) were a gift from Sandoz Pharmaceuticals (Dorval, Quebec). Propranalol.HCl and pronethalol.HCl were given to us by Ayerst, McKenna, Harrison. Dibenamine.HCl (in a solution of propylene glycol-ethanol) was obtained from Givaudan-Delawanna, Inc. (New York). Dibenzyline.HCl was a gift of the Department of Pharmacology, McGill University. Dichloroisoproterenol.HCl was donated by E. Lilly and Co. Tryptophol, 4-hydroxytryptophan, α-methyltryptamine were received from the Upjohn Co. The iboga alkaloids were sent by Geigy S.A. All other chemicals purchased were of optimum purity available. The materials were dissolved in water and injected at neutral pH. Hematin was generated by dissolving hemin in 0.01N NaOH.

3. Animals

Albino rats of Sprague-Dawley strain (90-180 g) were purchased from Canadian Breeding Laboratories at least one day before use. The specific weights are given with each experiment. The rats were fed standard Purina rat chow

except where noted.

B. Methods

1. Adrenalectomy

Mr. G.T. Berci demonstrated the technique of adrenalectomy and performed the operations in the earlier part of the work. The adrenals were excised from rats (90-110g) under ether anaesthesia by the lumbar route. One percent NaCl was substituted for drinking water and in some experiments deoxycorticosterone (1 mg/100 gm) was injected daily. The adrenalectomized animals were used 3-7 days after the operation.

2. Vitamin Deficiency

Rats, weighing 40 g, were fed a standard diet of Purina rat chow for three days. Following this they were grouped into sets of four by weight-matching and fed ad libitum a vitamin B_6-deficient diet in powder form (General Biochem.) for 4-6 weeks. The controls received the same diet but this was supplemented with 30 mg/kg of pyridoxine.

3. Copper-Deficiency Experiment

Male rats (Sprague-Dawley) were made copper-deficient by feeding a liquid diet low in copper. The animals (30 g) were divided into two groups and maintained on this diet for 91 days. The diet was Farmer's Wife No. 2 evaporated milk diluted 1 : 1 with water. Each rat was supplemented daily with Fe^{++} ($FeSO_4.7H_2O$) 0.4 mg, Mn^{++} (as $MnSO_4.4H_2O$) 0.05 mg, thiamine.HCl 0.05 mg, riboflavin-5-phosphate 0.05 mg, pyridoxine 0.01 mg, nicotinic acid 0.1 mg, Ca pantothenate 0.4 mg,

p-amino benzoic acid 0.1 mg, folic acid 0.02 mg, inositol 5 mg, choline.HCl 20 mg, vitamin B_{12} 0.5 ug, biotin 0.5 ug. Cu^{++} ($CuSO_4.5H_2O$), 0.05 mg, was given to each control animal daily.

Tissue copper levels were determined by the method of Eden and Green (200) as modified slightly by G. Gregoriadis of this laboratory.

4. Collection and Assay of $^{14}CO_2$

The method used was essentially that of Jeffay and Alvarez (201) as adapted by Moran and Sourkes (168). Rats (100-130 g) weight-matched to ± 10 g were injected intraperitoneally with the test compound (1-1.5 ml) followed by the radioisotope on the contralateral side (0.5 ml). Controls received 0.9% NaCl. Immediately afterwards, the animals were placed in glass metabolism cages through which CO_2-free air was constantly drawn (Fig. 6). The $^{14}CO_2$ was trapped from the atmosphere of each cage by a solution (50 ml) of ethylene glycol monomethyl ether: ethanolamine (2 : 1). Aliquots of the solution (3 ml) were removed hourly for 6 hours or less, mixed with phosphor (PPO[a] 0.55%, dimethyl POPOP[b] 0.01% in toluene, 15 ml) and the radioactivity was measured in a liquid scintillation spectrometer (Packard Inst. Co.). The counting efficiency was about 70%. Water quenching was minimized by; a) passing air through concentrated sulfuric acid before it entered the cages, b) submerging traps, located before the collecting tubes, into ice to precipitate expired

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[a]PPO - 2,5-diphenyloxazole

[b]POPOP - 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

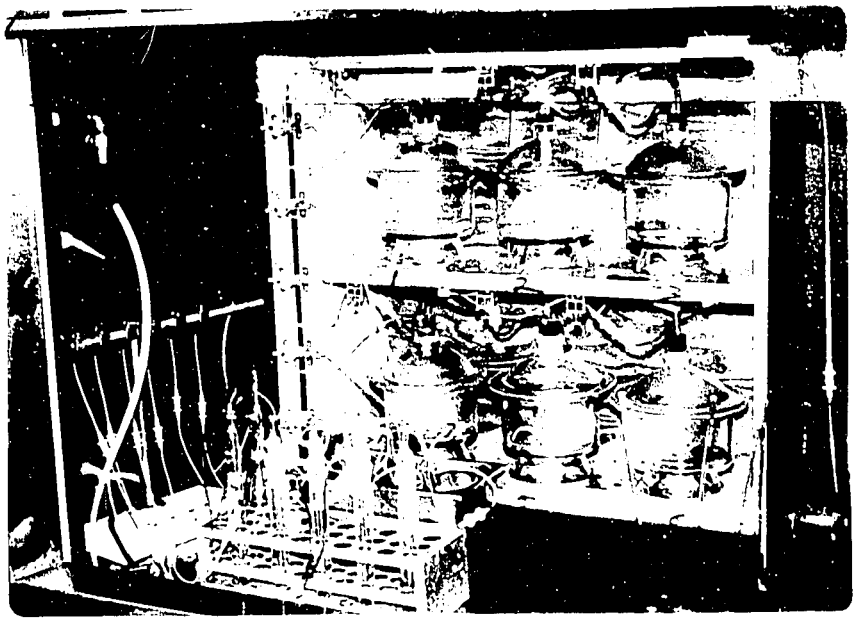


Fig. 6. Apparatus for collection of $^{14}CO_2$

water.

Results, obtained by averaging two 10 minute counts, are expressed as % ^{14}C recovered as $^{14}CO_2$, at a given time.

$$\% \ ^{14}C \text{ recovered as } ^{14}CO_2: \quad \frac{\left(CPM \times \frac{V}{3} \times \frac{CPM\ i.s.}{CPM_T - CPM}\right) + \sum CPM_R \times \frac{100}{1}}{CPM_I}$$

where: CPM - counts/min in a sample

V - volume remaining in collecting tube

$\frac{V}{3}$ - $\frac{\text{volume remaining}}{\text{aliquot volume}}$

$CPM_{i.s.}$ - internal standard counts/min (unquenched)

CPM_T - sample counts/min + internal standard counts/min

$(CPM_T - CPM)$ - quenched internal standard counts/min

CPM_R - counts/min previously removed from collecting tube

CPM_I - counts/min injected

5. Purification of ^3H-DL-α-methyltryptophan (^3H-AMTP)

The initial steps of the purification procedure were developed using non-radioactive material. Lyophilized ^3H-AMTP (30-40 mg) was dissolved in water; the solution was evaporated to dryness in order to remove some of the labile tritium. The crystals were redissolved in water and streaked across most of the width, 40 cm of Whatman No. 3 mm chromatography paper (46 cm x 57 cm) which had been previously washed with water. Standards of pure α-methyltryptophan were spotted at both edges. After descending chromatography in n-buta-

nol: acetic acid: water (4:1:5) the paper was dried and two small strips cut from both sides. One strip -- having the pure standard -- was sprayed with ninhydrin reagent (0.2% in acetone containing pyridine); the other -- radioactive -- strip was scanned in a Packard Automatic Windowless Scanner (Fig. 7). By this method the radioactive band corresponding to α-methyltryptophan was located, isolated and eluted with water. The eluate was evaporated to a small volume and subjected to paper chromatography four additional times. The final peak contained a bifurcation.

Fig. 7 shows the Rf values of ^{3}H-AMTP in each solvent system. Even after 48 hours of exposure to the final solvent, (80 cm of chromatography paper) the two peaks could not be resolved. Resolution of the D- and L-forms may have occurred. The ^{3}H-α-methyltryptophan was used as such. The concentration of ^{3}H-α-methyltryptophan in the final solution was measured by the ninhydrin method (202). Its activity was determined in a liquid scintillation spectrometer. An aliquot of the unknown solution was dissolved in Hydroxide of Hyamine (Packard Inst. Co.), phosphor (PPO 0.5%, POPOP 0.01% in toluene) and radioactivity determined. The efficiency of this system was 18.2%. The final specific activity of two different preparations were 2.02 mc/mM and 1.06 mc/mM.

6. Determination of Tryptophan and α-Methyltryptophan

a. Colorimetric Method

The method used was a modification of that described

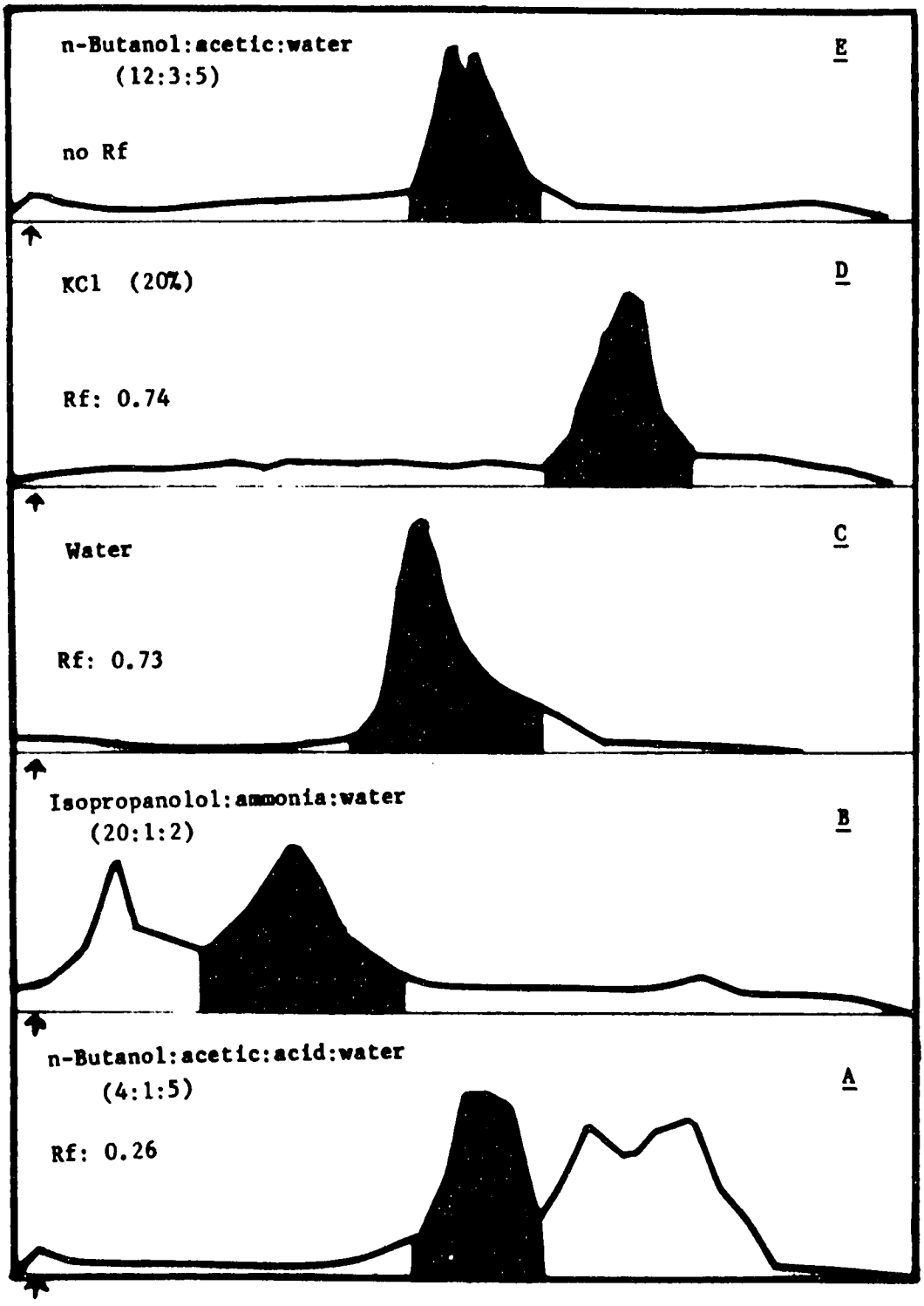


Fig. 7

(See legend p. 54)

Fig. 7. Stepwise purification of ^{3}H-DL-α-methyltryptophan by paper chromatography.

The shaded areas represent the peaks corresponding to ^{3}H-α-methyltryptophan. The ordinate indicates radioactivity and the abscissa, distance along the paper. The arrows point to the origin. Solvent systems and Rf values are shown on the upper left-hand corner. Purification proceeds from A-E.

by Dickman and Crockett (203).

Serum: Blood was removed from the tail vein or dorsal aorta of ether anaesthetized rats, and centrifuged twice to obtain clear serum. The serum (200 µl) was transferred to a tube containing 1% picric acid (0.5 ml) and ammonium sulfate (37 mg). After mixing and centrifuging at 3000 rpm for 30 minutes, the supernatant was filtered and the filtrate (400 µl) added to a solution of xanthydrol, (1 mg xanthydrol/5 ml conc. HCl (500 µl)), and glacial acetic acid (100 µl). The tubes were placed in a boiling water bath for 15 minutes, then cooled and NaOH (50 % w/v) was added until a pH of 3.5 was attained. Isoamyl acetate was used to extract the residual picric acid and xanthydrol. HCl — (6N, 200 µl) was added to the lower phase, and the absorbance of this solution was measured in a Coleman Junior Spectrophotometer at 500 mµ; linear range 4-80 µg, recovery 80-95%.

Tissues: Organs were rapidly removed and kept on ice or frozen until used. They were homogenized in ice-cold distilled water and processed the same way as serum, except that slightly different proportions of reagents were used.

b. Radioisotopic Method

Total tritium and ^{14}C in tissues was measured by a modification of the method of Herberg (204). Each organ was homogenized in ice-cold distilled water and then lyophilized. The lyophilysate (10 mg) was shaken with Hydroxide of Hyamine (Packard Inst. Co.) for one hour at 55°-60°C, in a counting

vial. At the conclusion of this step, phosphor (PPO 0.5%, POPOP 0.01%, in toluene) was added to the vial and radioactivity determined. Both Hyamine and the yellow colour produced by the dissolved tissue contributed to the quenching (32% - 57%). This was corrected for by (internal) standardization of each vial. The addition of hydrogen peroxide did not lessen the yellow colour, contrary to Herberg's findings. This method was also used to measure radioactivity in serum; no incubation was necessary.

To measure the radioactivity of the trichloroacetic-acid insoluble fraction, the lyophilized material (20 mg) was dissolved in water and trichloroacetic acid was added to a final concentration of 10%. The precipitate was resuspended in water and centrifuged. After several washings (5-10) the radioactivity of the precipitate reached a constant value. The precipitate was dried, dissolved in Hydroxide of Hyamine, and after addition of phosphor (as above) the radioactivity was determined for 90 minutes.

7. Measurement of Kynurenic and Xanthurenic Acids

Kynurenic and xanthurenic acids were measured in rat urine by two different methods. Several procedures for the extraction and determination of these quinaldic acids were examined before the final ones were selected, including paper and thin-layer chromatography, charcoal and Sephadex column chromatography, and solvent extraction.

a. Separation by paper chromatography

The method used was essentially that of Coppini et al. (205). Rats were placed in metabolic cages and urine was collected under toluene.

Standards (20 µg/20 µl) or urine (200-400 µl) were applied on Whatman No. 1 chromatography paper and run in 2 solvent systems: butanol: acetic acid; water (4:1:5) and water. The Rf's of kynurenic acid were 0.66, 0.68 and xanthurenic acid, 0.54, 0.47. Both acids were eluted from paper and measured by a colorimetric (xanthurenic acid) and spectrophotometric method (kynurenic acid). A linear relationship between absorbance and concentration was achieved in the range of 15-100 µg.

b. Separation by column chromatography

The method used was essentially that of Satoh and Price (206). Urine was applied to a Dowex 50-8X column. Kynurenic and xanthurenic acids were eluted from the column with water at gravity flow rates. Kynurenic acid was determined in an acidic medium using an Aminco-Bowman spectrophotofluorimeter. The activating wavelength was 340 mµ, the fluorescing, 435 mµ. Xanthurenic acid was determined at an alkaline pH. The activating wavelength was 370 mµ, the fluorescing 530 mµ.

8. Tryptophan Pyrrolase Assay

Tryptophan pyrrolase was measured in vitro by a modified (118) method of Knox et al. (157).

Rats were decapitated and exsanguinated. The livers were removed, rinsed in ice-cold water, blotted, divested of fibrous material, and weighed. Liver homogenate was made,

using a Teflon pestle and motor drive, in 0.14M KCl containing 0.005N NaOH. The final concentration was 12.5% or 25%. Aliquots of the homogenate were added to beakers containing Na phosphate buffer (0.2M, pH 7.0), hematin (5-7 x 10^{-6}M, final concentration), the test drug, and water to a final volume of 6 or 8 ml. (Cell sap was prepared by centrifuging the homogenate at 105,000 x g for 60 minutes.)

The reaction was started by the addition of tryptophan (1.7 x 10^{-3}M, final concentration) and stopped with metaphosphoric acid (15%). The blanks contained all the constituents except tryptophan. Optimal amounts of substrate and cofactor were used; the reaction rate was proportional to the enzyme concentration in the range of 1.5%-6%, final concentration. When drugs were added to the medium the pH was checked at the start of the experiment. The beakers were shaken in a Dubnoff metabolic shaker for 60 minutes (or 90 minutes for the inactivation studies) at 37°C. After adding metaphosphoric acid, the contents were filtered, an aliquot (2-3 ml) was removed and neutralized (pH 6.8-7.2) with NaOH. Kynurenine concentration was determined at 365 mμ in a Beckman DU spectrophotometer.

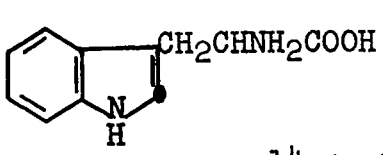
In drug-treated animals the livers from at least two similarly-treated animals were pooled.

C. Results

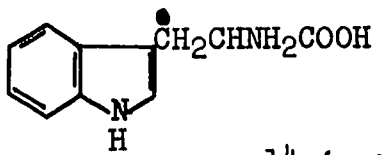
1. Metabolism of Tryptophan and ∝-Methyltryptophan in the Rat

a. Respiratory $^{14}CO_2$ from tryptophan labeled with ^{14}C in different positions

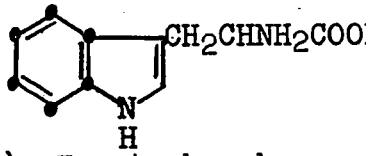
i. A comparison of the rate of evolution of $^{14}CO_2$ from DL-tryptophan-2-^{14}C, DL-tryptophan-3'-^{14}C, and DL-tryptophan-benzene-u-^{14}C (uniformly-labeled) is described.



Tryptophan-2-^{14}C(T2)



Tryptophan-3'-^{14}C(T3')



Tryptophan-benzen ^{14}C(TB)

The only known metabolic pathway which could result in the removal of the radioactive carbon from tryptophan labeled in these positions is the pyrrolase pathway. The amount of $^{14}CO_2$ produced from ^{14}C located in a specific position provides an index of the rate at which this carbon is cleaved enzymatically from the molecule.

It was considered important to determine the rate at which tryptophan is oxidized under physiological conditions. Other studies on the rate of evolution of $^{14}CO_2$ from tryptophan-^{14}C have been reported (23, 68, 91, 95, 168). But to date, the rate of oxidation of the radioisomer which would reflect most directly the activity the tryptophan pyrrolase (tryptophan-2-^{14}C), has not been determined. Also an estimate of the

degree to which the benzene ring (from tryptophan, uniformly-labeled in the benzene ring) is completely oxidized in a six hour period has not been reported. In later sections a comparison of the rates of metabolism of these radioisomers will be used to outline grossly the rate-limiting steps of the pathway for the whole animal and to detect multiple sites of drug effects.

All three forms of radioactive tryptophan are oxidized at approximately the same rate (Table I, Fig. 8). A more careful study of the values (Table I) points to the following conclusions:

1) Within 6 hours of injection, a maximum of 10.7% of exogenous tryptophan (0.2 mg/100 g) is oxidized via the pyrrolase pathway. The values obtained using T_2 and $T_{3'}$ are in close agreement, and are somewhat lower than those using T_B. It is conceivable that the breakdown products of the benzene ring of tryptophan, derived from 3-hydroxyanthranilic acid, enter fewer diluting pools than those from T_2 and $T_{3'}$. Therefore, although the oxidative rate appears to be slightly faster, this could be more apparent than real.

2) Although tryptophan pyrrolase activity is lowered slightly in adrenalectomized animals (122, 143, 157), there is no difference in the apparent rates of oxidation of tryptophan by intact and adrenalectomized animals, in vivo.

There is a greater variability (using T_2 and $T_{3'}$) in unoperated rats, as reflected in the larger standard deviations

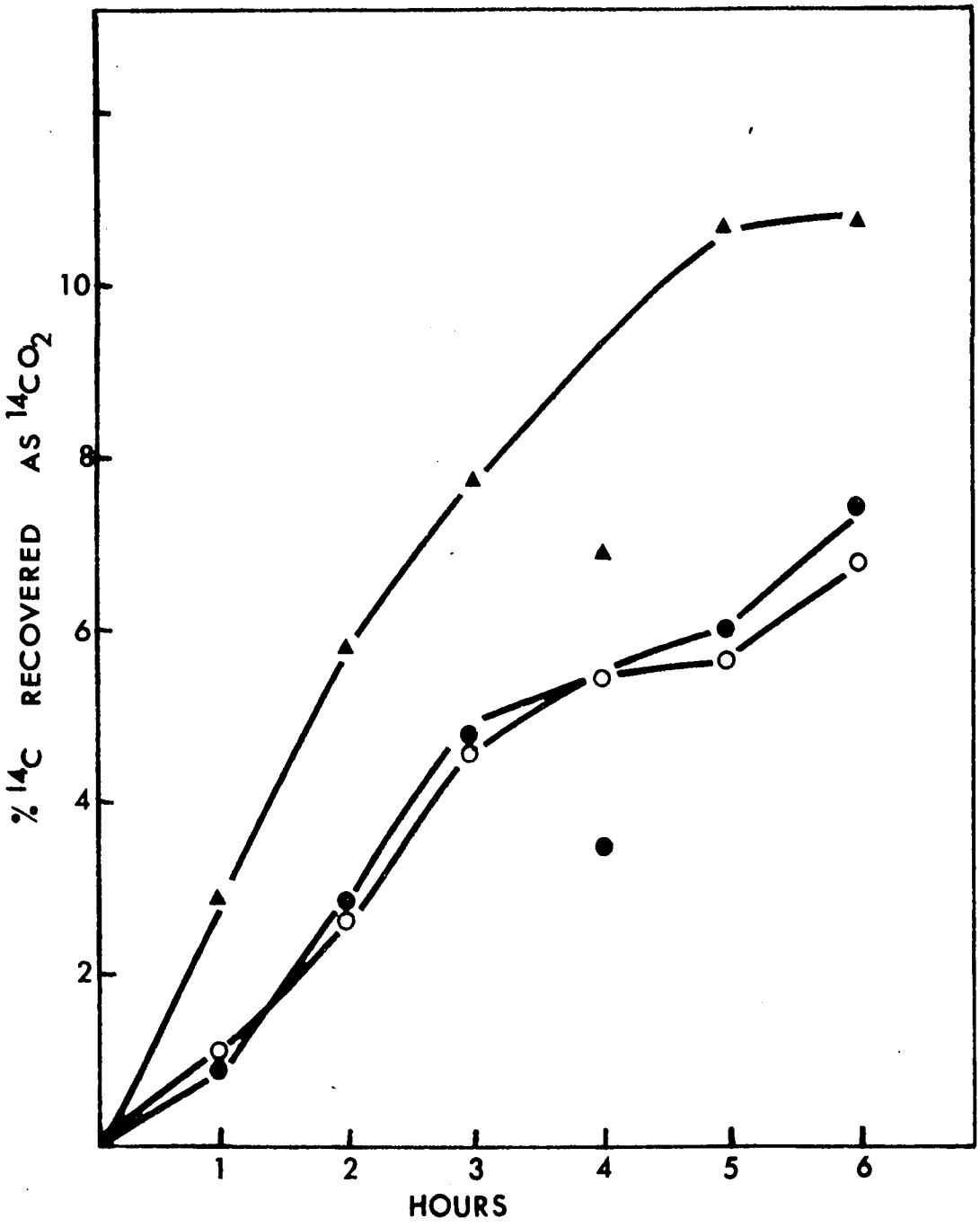


Fig. 8. The rate of formation of $^{14}CO_2$ from DL-tryptophan labeled with ^{14}C in different positions.

Rats were injected intraperitoneally with DL-tryptophan-2-^{14}C (● - ●), DL-tryptophan-3'-^{14}C (○ - ○) and DL-tryptophan-benzene-u-^{14}C (▲ - ▲). See Table I for the dose. Results are expressed as the % of radioactivity injected that was recovered as $^{14}CO_2$.

of these two groups. The most likely explanation is that tryptophan pyrrolase activity is subject to wider fluctuations in the intact animal owing to a variable rate of secretion of glucocorticoids, a process that is absent in the adrenalectomized rat. After adrenalectomy the $^{14}CO_2$ formed from T_2 is less variable than that from T_3, or TB (2-6 hours).

The effect of body weight on $^{14}CO_2$ production was determined; in the weight range of 100-150 g there was no effect of weight. Rats weighing in excess of 250 g expired more $^{14}CO_2$ than smaller animals -- for the first few hours -- but at the end of 6 hours the values reached were the same. This is probably due to the volume effects in the cages. With increasing animal size, the dead space in the apparatus decreases, resulting in a faster turnover of air; this leads to more rapid accumulation of the initial expired $^{14}CO_2$. But, the net amount expired is the same, because for equimolar amounts of tryptophan the same proportion is oxidized to $^{14}CO_2$ regardless of body sizes.

ii. Oxidation of DL-tryptophan-^{14}COOH and L-tryptophan-^{14}COOH

The carboxyl group on the side chain of tryptophan can be converted to CO_2 by several routes: 1) decarboxylation of tryptophan, 5-hydroxytryptophan or indolepyruvic acid, or 2) by the action of pyruvic kinase on pyruvic acid formed after transamination of the alanine side chain of tryptophan.

It was considered pertinent to study the rate of forma-

Table I Comparison of the rate of oxidation of DL-tryptophan-2-^{14}C, DL-tryptophan-3'-^{14}C and DL-tryptophan-benzene-^{14}C $\longrightarrow$ $^{14}CO_2$ in vivo.

Intact (i) or adrenalectomized (a) rats were injected intraperitoneally with one of the three isomers of tryptophan (1.08 mc/mM). They also received 0.9% NaCl by the same route. Results are expressed as mean ± S.D. (average of 7 animals).

Time after injection		% of radioactivity injected which was recovered in respiratory $^{14}CO_2$ from:		
		TRYPTOPHAN-2-^{14}C	TRYPTOPHAN-3'-^{14}C	TRYPTOPHAN-b-[1]
1 hr	(i)	0.9 ± o.3	1.2 ± 0.7	2.9 ± 1.0
	(a)	1.1 ± 0.3	0.9 ± 0.3	2.2 ± 0.9
2 hr	(i)	2.8 ± 2.3	2.7 ± 1.3	5.8 ± 1.4
	(a)	3.0 ± 0.3	2.5 ± 0.7	4.6 ± 1.3
3 hr	(i)	4.7 ± 2.8	4.6 ± 1.8	7.7 ± 1.9
	(a)	4.7 ± 0.8	4.0 ± 1.0	6.6 ± 1.8
4 hr	(i)	3.5[b]	5.2[b]	6.9[b]
	(a)	5.8 ± 0.7	5.2 ± 1.1	7.2 ± 2.4
5 hr	(i)	6.0 ± 2.7	5.6 ± 2.0	10.6[b]
	(a)	6.7 ± 0.9	6.3 ± 1.4	9.2 ± 2.6
6 hr	(i)	7.6 ± 3.1	6.7 ± 2.1	10.7 ± 1.9
	(a)	7.8 ± 0.9	7.1 ± 1.4	9.5 ± 3.1

[b] 3 animals

tion of $^{14}CO_2$ from carboxyl-labeled DL- and L- tryptophan. The extent to which D-tryptophan is metabolized in a short period of time can be estimated in this way.

Assuming that direct decarboxylation of tryptophan or its indole derivatives occurs to a very small extent, the rate

of formation of $^{14}CO_2$ from this radioisomer would be the most sensitive indicator of the activity of the pyrrolase pathway, because of minimum dilution of the radioactive product.

Table II. Formation of respiratory $^{14}CO_2$ from DL- and L-tryptophan -^{14}COOH in adrenalectomized rats.

Rats were injected intraperitoneally with either DL-tryptophan-^{14}COOH (1.08 mc/mM, 0.145 mg) or L-tryptophan-^{14}COOH (1.08 mc/mM, 0.145 mg) to which an equivalent amount of D-tryptophan was added. Results are expressed as mean ± S.D. (4 animals).

	% $^{14}CO_2$ RECOVERED AT:		
	2 hrs	4 hrs	6 hrs
DL-TRYPTOPHAN-^{14}COOH	5.2 ± 1.4	8.7 ± 1.4	11.2 ± 1.7
L-TRYPTOPHAN-^{14}COOH	6.2 ± 1.6	9.6 ± 1.7	12.2 ± 1.6

The results (Table II) indicate that the rate of formation of $^{14}CO_2$ from DL- and L-tryptophan-^{14}COOH is essentially the same, although the mean values derived from the L- form are slightly higher. It can be concluded that D-tryptophan is rapidly transaminated to indolepyruvic acid. It may be decarboxylated at this point (24) or the keto acid can be converted to L-tryptophan and then give rise to $^{14}CO_2$ from the pyrrolase pathway. The contribution of the D- form to respiratory $^{14}CO_2$ probably stems from its metabolism along the pyrrolase route. Only a very small amount of indole-3-acetic acid (a source of which is indolepyruvic acid) can be recovered from administered tryptophan (24).

The rate of formation of $^{14}CO_2$ from these radioisomers of tryptophan was higher than that from the labeled tryptophan studied in the previous section (p.63). This is probably due to the rapid decarboxylation of pyruvate-$^{14}COOH$ to $^{14}CO_2$, following the action of tryptophan pyrrolase, formamidase, kynurenine hydroxylase, kynureninase and alanine transaminase.

b. i. Accumulation of α-methyltryptophan in serum and tissues.

Our laboratory has shown that a single injection of α-methyltryptophan causes an induction of tryptophan pyrrolase lasting as long as a week (168, 169). It is not known whether the inducing agent remains in the body during this period, thus accounting for the elevated enzyme activity. It was therefore pertinent to determine for what period of time the amino acid analogue could be detected.

This was done by studying its accumulation and disappearance in various organs. The distribution of tryptophan was also followed and compared with that of the synthetic analogues.

Rats, injected with L-tryptophan or DL-α-methyltryptophan.H_2O (1 mmole/kg), were killed at different time intervals and the xanthydrol-reacting material was measured. Both amino acids entered the blood stream rapidly and reached peak concentrations within one hour (Fig. 9). L-tryptophan disappeared rapidly and excess amounts were undetectable within 6 hours.

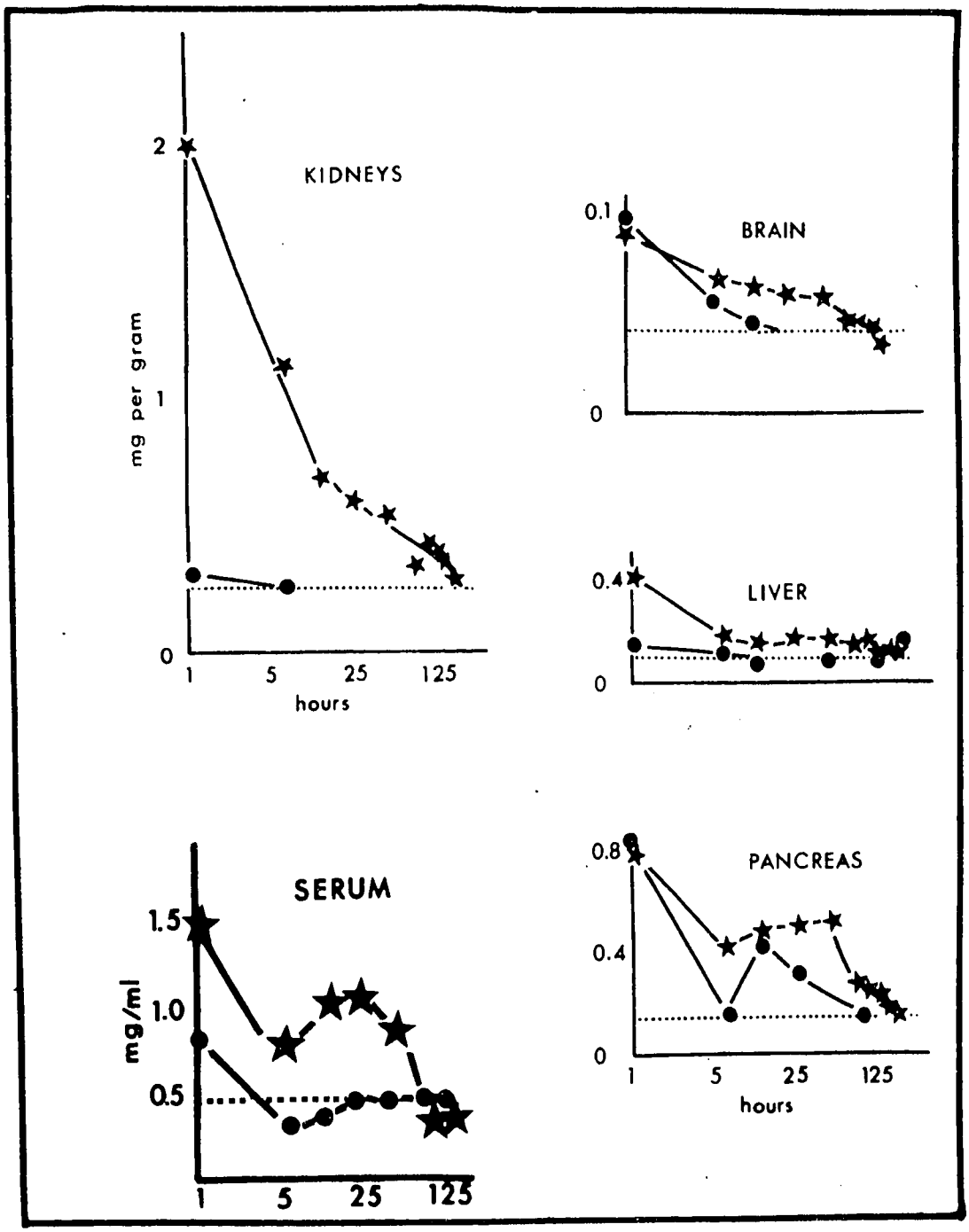


Fig. 9. Concentration of tryptophan and α -methyltryptophan in serum and organs of rats.

Male rats were injected intraperitoneally with L-tryptophan (1 mmole/kg) ● - ●, and DL-α-methyl-tryptophan.H_2O (1 mmole/kg) ★ - ★ ; controls (- - -) received 0.9% NaCl. The ordinate denotes the concentration of the amino acids as mg/ml (serum) or mg/g (wet weight of tissue). The abscissa indicates the time after injection, on a logarithmic scale.

On the other hand, DL-∝-methyltryptophan remained in the serum for a much longer period of time.

The kidneys accumulated and retained high concentrations of indoles following injection of ∝-methyltryptophan, the excess levels persisting for almost a week. Indolic substances attained normal levels in the kidney within 5 hours of tryptophan administration.

The entry of xanthydrol-positive material into the brain was not as marked as in other organs but there nevertheless were increases following injection of both amino acids. The amino acid analogue persisted in this tissue for several days.

∝-Methyltryptophan and/or its indolic metabolites were detectable in the liver for more than four days. On the other hand, excess tryptophan disappeared within twelve hours. As tryptophan induces its degrading enzyme in the liver it is not surprising to see a rapid fall of tryptophan levels in this organ.

The pancreas showed high concentrations of both amino acids within the first hours after injection, the concentrations fell to a low point at 6 hours, and then rose again providing new peaks. In this connection, the pancreas is recognized as a site of rapid protein synthesis, and a rapid uptake of amino acids can be expected in that organ.

The phenomenon resembles the accumulation by the pancreas of another synthetic amino acid, 1-aminocyclopentanecarboxylic acid, as reported by Berlinguet and his colleagues (207). This

amino acid, like α-methyltryptophan, lacks an α-hydrogen, a fact that limits the number of possible routes of metabolism. Although 1-aminocyclopentanecarboxylic acid is rapidly taken up, it is not incorporated into pancreatic protein. The secondary rise in the concentration of amino acids may represent accumulation of the compounds being released from other organs where they had been absorbed earlier. It is interesting to note that the pancreas accumulated the most tryptophan on a weight basis and retained it for the longest period of time.

ii. Distribution of ^{3}H-DL-α-methyltryptophan

Because of the unusually long residence of α-methyltryptophan and its metabolites in the body, as observed in the above experiments, it was decided to investigate the distribution of α-methyltryptophan further by employing the more sensitive method of tracing with a small amount of radioactive material. Despite the discrepancy in the weights of the animals and in the doses of α-methyltryptophan (unlabeled material: tritiated, 15:1) both tracing techniques agreed well. ^{3}H-DL-α-Methyltryptophan was accumulated in decreasing amounts by the kidneys, pancreas, intestine, spleen, heart, liver, lung and brain within the first 6 hours (Fig. 10). At 24 hours, the levels in the kidneys and pancreas were one-half the earlier values; less significant changes were noted in other organs. At the end of five days very low activity was detected in the kidneys, pancreas, intestine, liver and lungs. This does not eliminate the possibility that the radioactive amino acid was

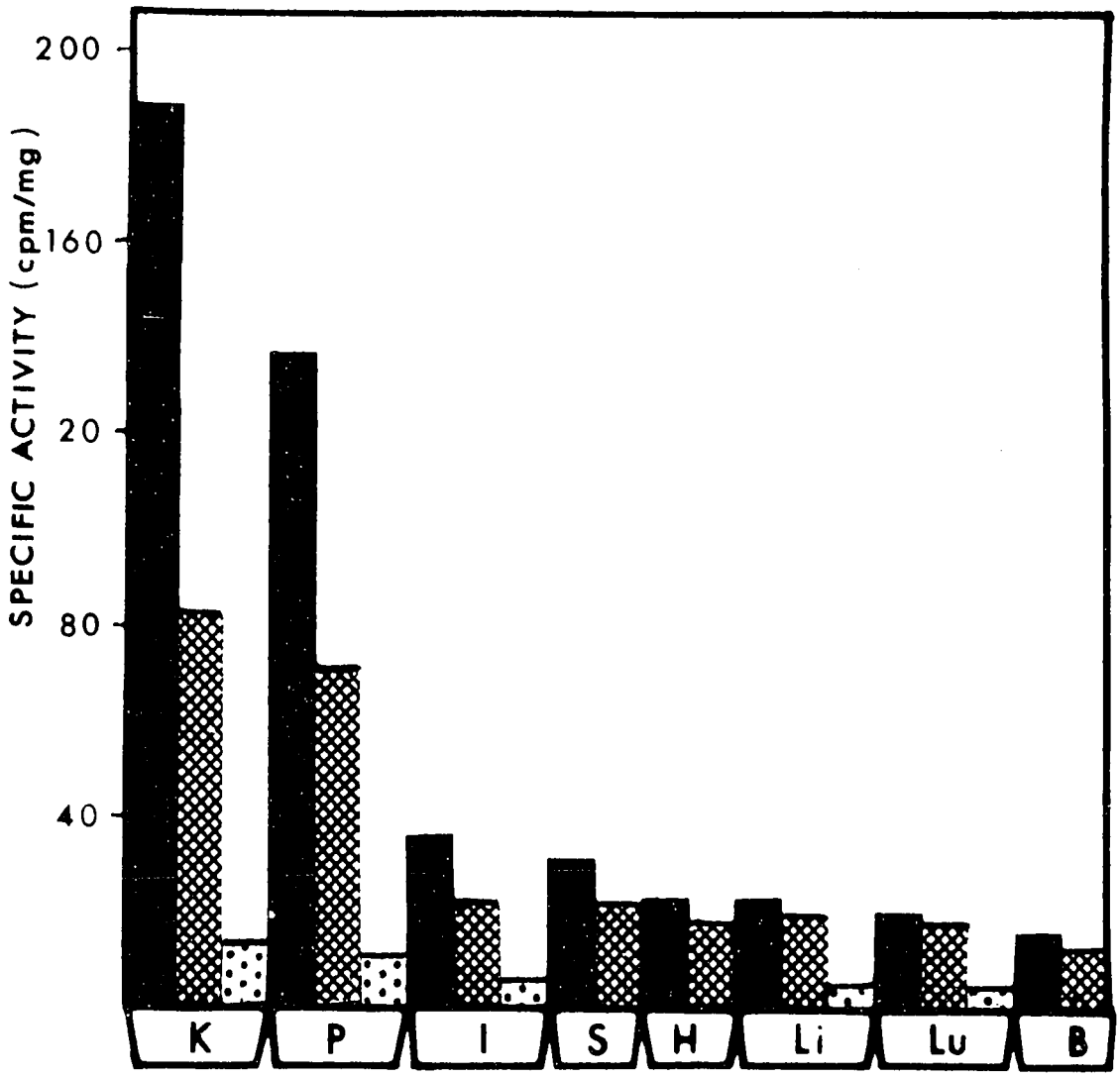


Fig. 10. The measurement of ^{3}H-DL-α-methyltryptophan in organs.

Three male rats (60-62 g) were injected with 8 μc of ^{3}H-DL-α-methyltryptophan and killed at 6 hours (solid bar), 24 hours (cross-hatched bar), 5 days (dotted bar). The ordinate indicates specific activity of each organ expressed as cpm/mg dry weight of tissue. The organs are: K, kidneys; P, pancreas; I, intestine; S, spleen; H, heart; Li, liver; Lu, lung; B, brain.

present in other tissues, but, if so, in amounts beyond the limits of detectability by this method.

The results of these experiments were compared to the data obtained after injecting unlabeled material.

The comparison is shown in Table III. The concentration of the xanthydrol-positive material and the radioactivity, respectively, in pancreas, brain and kidneys are expressed as ratios of the values found for liver. The closest agreement in results was observed at 24 hours. The greater discrepancies at 6 hours may be accounted for by the rapid changes in concentration taking place at this period (Fig. 9) and the fact that the value for labeled material is based on but one animal per time period. The tritiated compound was more concentrated by the pancreas and kidney (with respect to the liver) than the unlabeled material for all time periods.

The total recovery of radioactivity at 24 hours was 23.4%. The acid-insoluble fraction of the liver, pancreas and intestinal tract (of the rat killed at 6 hours) was also examined for radioactivity and very low values were obtained of the order of 10-40 cpm above background (Table IV). It has not been determined whether this activity represents residual contamination, or tritium-exchange, or whether it stems from a small but definite incorporation of α-methyltryptophan into protein.

Table III. Concentration of ∝-methyltryptophan in rat organs.

Three rats (60-62 g) were injected with ^{3}H- ∝-methyltryptophan as described in the text. The radioactivity found at the stated times in their organs is given as a ratio (liver, 1.0). Twelve rats were injected with one mMole of unlabeled ∝-methyltryptophan per kg., and indolic material was determined on the extracted organs, using a xanthydrol reagent (see "Methods"). Figures are ratios, relative to the value found for liver.

Time after injection	Organ	Experiments with ∝-methyltryptophan	
		tritiated	unlabeled
6 hours			
	Pancreas	6.8	2.4
	Kidney	9.0	5.6
	Brain	0.7	0.7
24 hours			
	Pancreas	3.0	1.7
	Kidney	4.0	3.4
	Brain	0.6	0.6
5 days			
	Pancreas	6.1	1.0
	Kidney	6.0	3.3
	Brain	0.3	0.2

Table IV. The radioactivity in the acid-soluble and acid-insoluble fractions of specific organs.

One rat was killed 6 hours following injection of ^{3}H-DL-α-methyltryptophan (8 µc); the supernatant and precipitate of tissues resulting from trichloroacetic acid treatment were examined for radioactivity, as discussed under "Methods". (Counting time: 20 minutes and 90 minutes, resp.)

ORGAN	ACID-SOLUBLE	ACID-INSOLUBLE
Liver	207 cpm	19 cpm
Intestine	316 cpm	31 cpm
Brain	141 cpm	10 cpm
Pancreas	1367 cpm	12 cpm

It can be concluded from these studies that: 1) the metabolism of tryptophan differs considerably from its α-methyl analogue, following injection of equimolar amounts of the two amino acids, the synthetic compound is present in some organs at higher concentrations and persists for a longer period of time; 2) α-methyltryptophan, and/or its metabolites can be detected in liver for at least 5 days, an observation that correlates very well with the time course of its induction of tryptophan pyrrolase.

2. Influence of High Doses of Administered L-Tryptophan and DL-α-Methyltryptophan on Tryptophan Metabolism

a. Effects on Tryptophan-^{14}C Oxidation in Intact and Adrenalectomized Rats

It is well established by *in vitro* methods that both tryptophan (21) and α-methyltryptophan (139) induce tryptophan pyrrolase. Recently Moran and Sourkes (168) showed that the induction could be detected in intact rats by injecting the inducing agent along with tryptophan-^{14}C and measuring the $^{14}CO_2$ produced. Under these conditions the amount of $^{14}CO_2$ expired was 2-6 times greater than control values. Having determined this, they traced the time course of the induction with both amino acids *in vivo*; the tryptophan-response wore off within 14 hours whereas α-methyltryptophan exerted an effect for as long as 7 days.

The objectives of this study were: 1) to verify the time course of the substrate-type induction in both intact and adrenalectomized rats. This would reveal the contribution of a stress mechanism to the increased rate of tryptophan catabolism and would resolve the issue as to whether the prolonged induction by α-methyltryptophan is mediated via the adrenal glands; 2) to compare the rates of metabolism of the different forms of radioactive tryptophan following induction by either amino acid. By injecting a large dose of tryptophan and then measuring the rates of oxidation of tryptophan labeled in different positions, that section of the degradative pathway which is rate-limiting *in vivo* can be detected. Moreover,

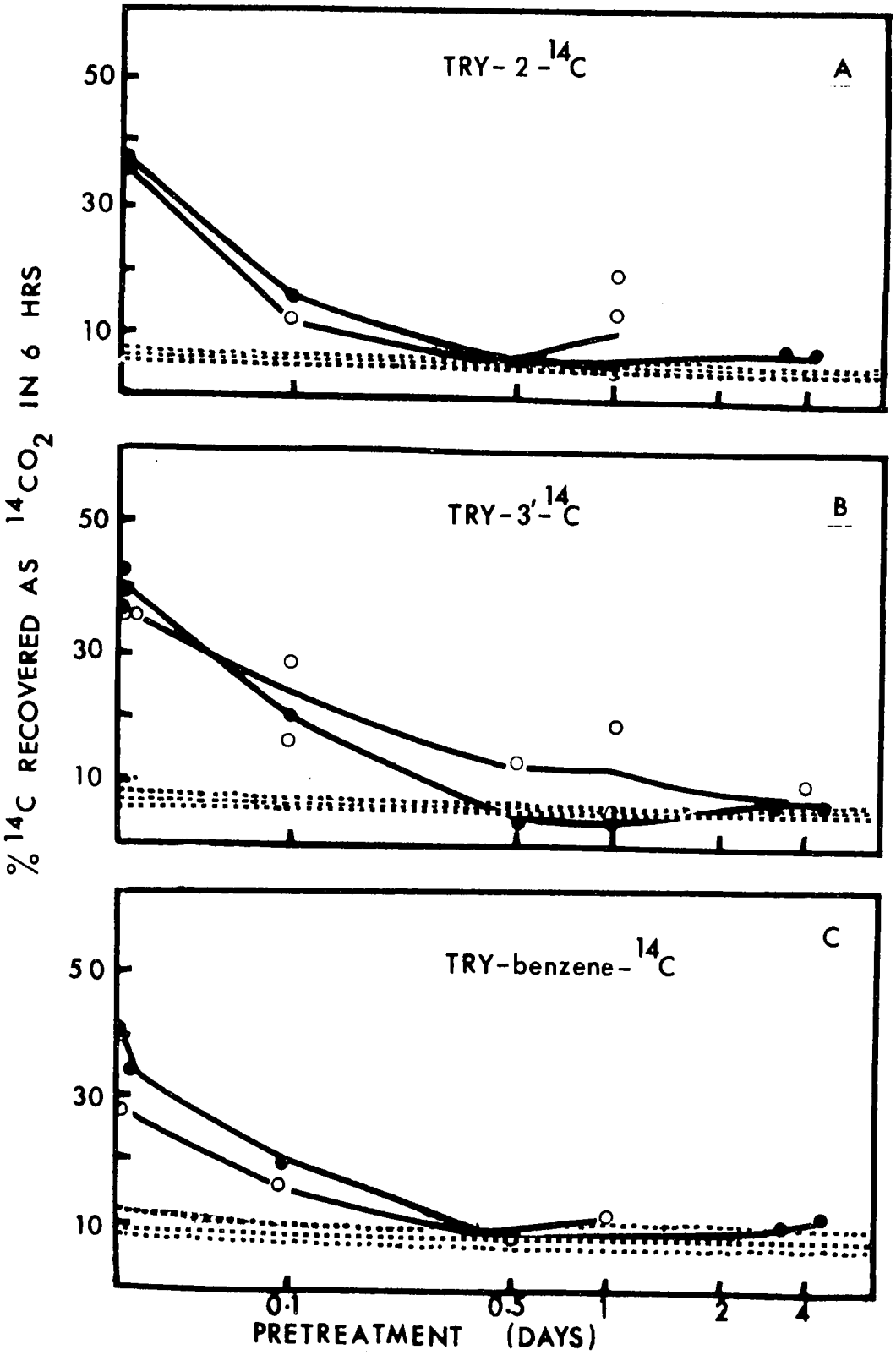


Fig. 11

(See legend p. 75)

Fig. 11. The effect of time of pretreatment with L-tryptophan-^{12}C on the evolution of $^{14}CO_2$ from DL-tryptophan-^{14}C.

Intact (● - ●) or adrenalectomized (o - o) rats were pretreated with L-tryptophan (1 mmole/kg) at the times indicated. Controls (- - - -) received 0.9% NaCl. DL-Tryptophan-2-^{14}C (A), DL-tryptophan-3'-^{14}C (B) or DL-tryptophan-benzene-^{14}C were injected intraperitoneally after the tryptophan load. (See Table V for dose.) The time of pretreatment is indicated on a logarithmic scale.

one can estimate the degree to which tryptophan is diverted into paths not part of the main metabolic route.

i. Effect of L-tryptophan

The curves illustrated in Fig. 11 are derived from values shown in Table V. In both intact and adrenalectomized animals, the highest amount of $^{14}CO_2$ (37.0-44.0%) was recovered when unlabeled tryptophan was injected at the same time as the radioactive material (Fig. 11). If a large dose of tryptophan was given 3 hours before the labeled compound about one-half of the maximal values were recovered. After 14 hours (and as long as 6 days) there was no evidence of residual induction by the tryptophan load.

In adrenalectomized animals, the induction appeared to be lower than in intact animals; for example, at 0 pre-treatment time, the % $^{14}CO_2$ recovered in intact rats from T_2, T_3', T_B was 41.7%, 40.6%, 39.4% (resp.) at 6 hours whereas in adrenalectomized rats the corresponding values were 33.9%, 36.3%, 23.8% (average of 2 experiments). This is in accord with the fact that an injection of a large amount of tryptophan is a stress (122). In contrast to unoperated rats, an accelerated oxidation was perceived 24 hours after treatment in 4 of 6 operated animals.

The induction was reflected equally by T_2, T_3', and T_B in intact rats. A comparison of the rate of oxidation of all forms of tryptophan-^{14}C, administered along with a large dose of unlabeled material, in adrenalectomized rats, is given in Fig. 12. T_2 and T_3' served to detect the induction generated

Table V. The effect of a tryptophan load on the oxidation of DL-tryptophan-2-^{14}C, DL-tryptophan-3'-^{14}C and DL-tryptophan-benzene-^{14}C in intact and adrenalectomized rats.

Animals were pretreated with L-tryptophan (1 mM/kg) at different times. The isotope (1.08 mc/mM/0.8 μc) was given just prior to $^{14}CO_2$ collection.

(A) TRYPTOPHAN-2-^{14}C

PRETREATMENT TIME[a]	% ^{14}C recovered as $^{14}CO_2$ at:					
	2 hrs.		4 hrs.		6 hrs.	
	INTACT	ADREN.	INTACT	ADREN.	INTACT	ADREN.
0	17.8	7.9	37.8	19.2	38.1,45.3	31.0,36.7
3 hrs.	7.7	6.8	12.4	10.4	15.5	12.0
14 hrs.	0.8	3.1	2.5	5.5	4.1	7.3
1 day	2.9 2.6 1.9	8.2 2.7 13.1	4.9 5.4 5.3	11.7 4.6 14.3	6.2 7.5 7.9	14.0 6.4 21.2
3 days	5.8	-	10.2	-	12.5	-
5 days	6.6	-	9.3	-	11.7	-
6 days	3.8	-	6.8	-	12.3	-
CONTROL[b]	2.8±2.3	3.0±0.3	3.9±1.6	5.8±0.7	7.6±3.1	7.8±0.9

(B) TRYPTOPHAN-3'-^{14}C

0	19.5 18.1	12.4 6.6	- -	34.6 26.6	37.0 44.2	36.6 36.0
1 hr.	-	13.7	-	-	-	36.2
3 hrs.	8.9	22.4 14.8	16.4	26.9 -	20.2	28.8 15.7
14 hrs.	0.8 -	11.3 3.4	2.0 -	18.7 5.1	3.7 -	21.4 6.1
1 day	1.0 -	2.9 14.1	2.9 -	3.8 -	4.7 -	7.5 20.0
3 days	3.5 4.7	3.3	6.6 11.2	6.6	7.4 14.9	7.4
4 days	4.1	3.4	7.4	6.8	9.8	8.9
5 days	4.1	-	-	-	8.1	-
6 days	2.3	-	4.3	-	8.8	-
CONTROL[b]	2.5±1.5	2.5±0.7	5.4±1.9	6.0±2.1	5.6±2.2	7.6±2.9

Table V. (cont'd)

TRYPTOPHAN-benzene-u-^{14}C

(C)	% ^{14}C recovered as $^{14}CO_2$ at:					
PRETREATMENT TIME[a]	2 hrs.		4 hrs.		6 hrs.	
	INTACT	ADREN.	INTACT	ADREN.	INTACT	ADREN.
0	14.9	4.0	34.7	-	37.3 41.4	29.6,18
1 hr.	15.1	-	-	-	33.0	-
3 hrs.	14.1	9.9	18.9	15.5	21.2	17.7
14 hrs.	3.6	-	5.1	-	6.4	-
1 day	-	7.9	-	-	-	13.5
3 days	6.1	-	-	-	11.2	-
5 days	6.9	-	10.4	-	12.3	-
CONTROL[b]	5.8±1.4	4.6±1.3	6.9-	7.2±2.4	10.7±1.9	9.5±3.1

[a] Refers to the time the tryptophan load was given, before the radioisotope.
[b] Mean ± S.D. (7 animals)

by tryptophan equally and concurrently. Therefore the steps leading from tryptophan to 3-hydroxyanthranilic acid formation are not rate-limiting, a result documented in the literature by other methods. Moreover kynurenine transaminase does not appear to divert significant amounts of kynurenine and 3-hydroxykynurenine away from the main degradative route. Results obtained with the benzene-labeled material were somewhat lower, but this difference may not be significant in view of the fact that only two animals were used. Nevertheless they merit further consideration because the control values

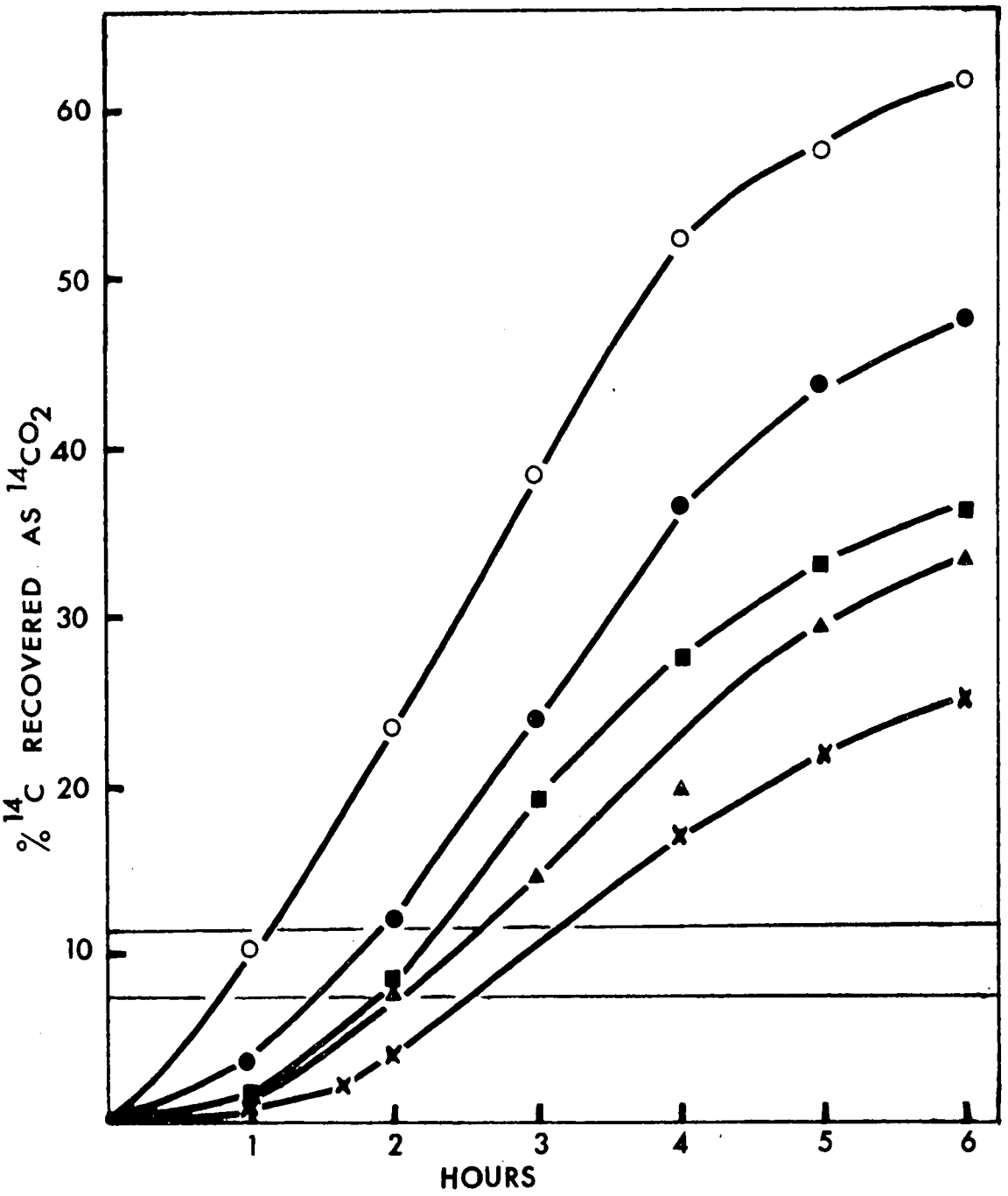


Fig. 12. The induction of tryptophan pyrrolase by L-tryptophan as measured by the formation of respiratory $^{14}CO_2$ from DL- or L-tryptophan-^{14}C in adrenalectomized rats.

L-tryptophan (1 mmole/kg) was injected intraperitoneally into adrenalectomized rats, followed by DL-tryptophan-benzene-^{14}C (X - X), DL-tryptophan-2-^{14}C (▲-▲), DL-tryptophan-3'-^{14}C (■-■), DL-tryptophan-$^{14}COOH$ (●-●) or L-tryptophan-$^{14}COOH$ (○-○). Controls (═) received 0.9% NaCl.

Each curve represents one animal

are at least as great as those obtained with the other forms of tryptophan. The lower $^{14}CO_2$ production may be a consequence of several factors: dilution of the radioactive material with corresponding metabolites (to be dealt with shortly), saturation of a rate-limiting enzyme, siphoning of excess products into side paths, or product inhibition.

The $^{14}CO_2$ produced from DL- and L-tryptophan-^{14}COOH was also measured in rats which had been treated with a tryptophan load; the label appeared in respiratory gases at a rate faster than from T_2, $T_{3'}$, or T_B (Fig. 12). This difference is probably more apparent than real because of the higher control values observed with this radioisomer. Thus the ratio of tryptophan-treated: control values (% ^{14}C recovered at 6 hr.) with T_2 or $T_{3'}$ is about 6:1 and with carboxyl-labeled L-tryptophan, 5:1. It is interesting to note that DL-tryptophan-^{14}COOH was not metabolized as rapidly as L-tryptophan-^{14}COOH, under conditions of tryptophan loading despite the fact that untreated animals metabolized the DL- and L- forms at equivalent rates.

ii. Effect of DL-α-methyltryptophan

The curves illustrated in Fig. 13 are derived from values outlined in Table VI.

The time curves (Fig. 13) confirm the findings of Moran and Sourkes (168) that, in α-methyltryptophan-treated animals, the peak activity of tryptophan pyrrolase is reached

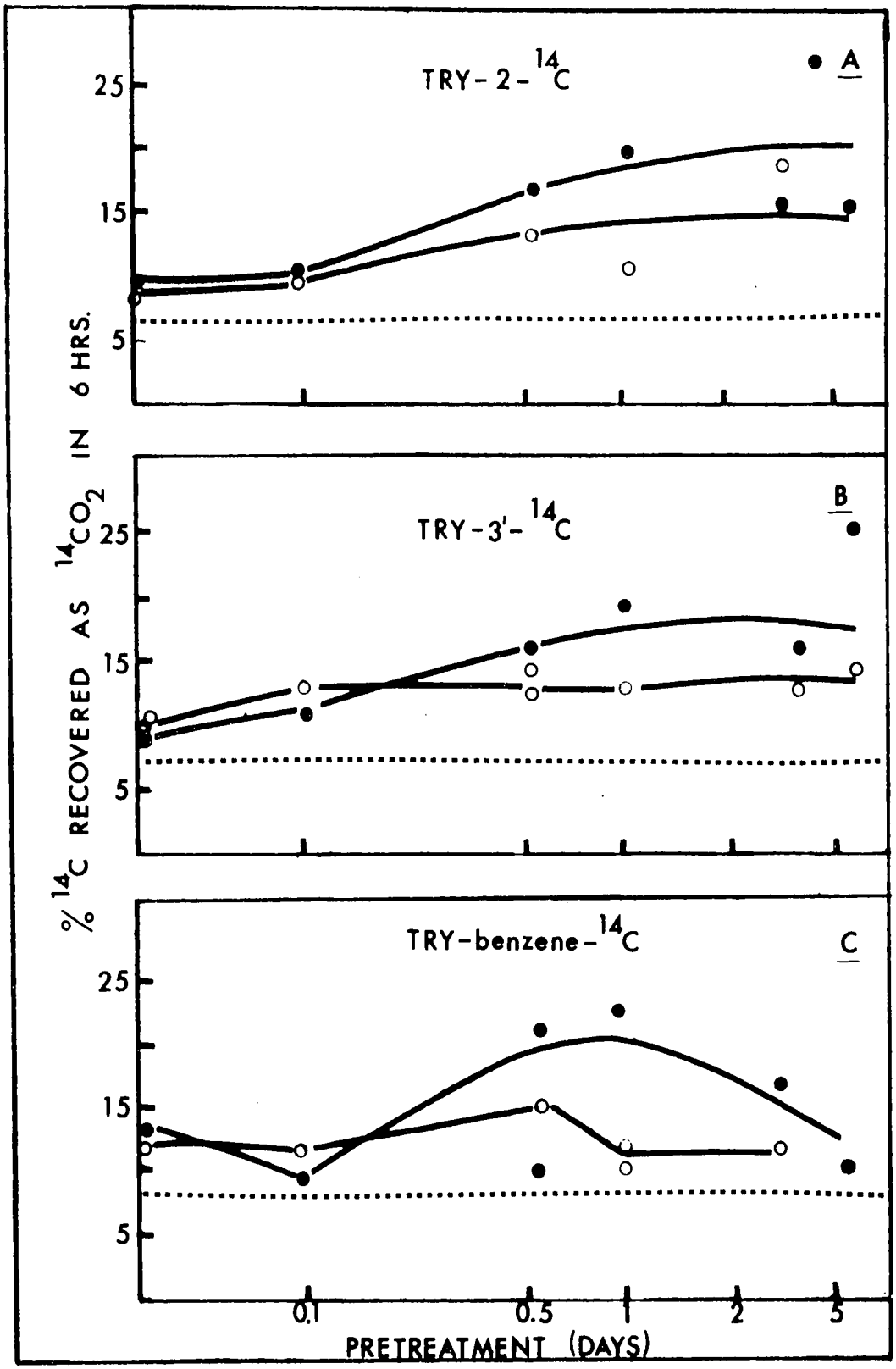


Fig. 13

(See legend p. 82)

Fig. 13. The influence of pretreatment with DL-α-methyltryptophan.H_2O on the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C.

Intact (●-●) or adrenalectomized rats (o-o) were pretreated with DL-α-methyltryptophan.H_2O (1 mmole/kg) at various times. Controls received 0.9% NaCl, (----). At the end of the pretreatment time, the rats were injected intraperitoneally with the form of DL-tryptophan-^{14}C, indicated. (See Table VI for dose.) The abscissa denotes time of pretreatment, logarithmically.

Table VI. The effect of DL-α-methyltryptophan on the oxidation of DL-tryptophan-2-^{14}C, DL-tryptophan-3'-^{14}C, or DL-tryptophan-benzene-^{14}C in intact and adrenalectomized rats. Animals were pretreated with DL-α-methyltryptophan (1 mmole/kg) at different times. The isotope (1.08 mc/mmole, 0.8 μc) was given just prior to $^{14}CO_2$ collection.

TRYPTOPHAN-2-^{14}C

(A)

PRETREATMENT TIME[a]	% ^{14}C recovered as $^{14}CO_2$ at:					
	2 hrs.		4 hrs.		6 hrs.	
	INTACT	ADREN.	INTACT	ADREN.	INTACT	ADREN.
0	3.6	5.3	6.1	7.2	8.9	8.8
3 hrs.	3.6	5.9	7.4	-	9.8	9.6
14 hrs.	6.5	6.3	12.6	9.9	16.7	13.4
1 day	5.6	4.6	14.6	8.3	19.8	10.6
3 days	7.7	9.0	11.1	14.4	14.0	17.5
5 days	13.9	-	23.2	-	27.6	-
6 days	5.8	-	9.6	-	15.8	-
CONTROL[b]	2.8±2.3	3.0±0.3	3.9±1.6	5.8±0.7	7.6±3.1	7.8±0.9

(B) TRYPTOPHAN-3'-^{14}C

0	2.9	3.7	-	7.4	7.7	9.0
1 hr.	-	6.0	-	-	-	12.4
3 hrs.	4.2	3.9	7.9	11.2	10.6	13.7
14 hrs.	6.3 -	5.8 9.8	11.8 -	9.6 12.8	15.7 -	12.2 14.4
1 day	6.7	6.2	14.4	-	18.8	12.3
3 days	7.3 8.7	- 5.1	11.9 14.6	- 8.9	14.4 17.9	- 12.2
4 days	6.4	5.2	10.4	7.7	12.4	9.9
5 days	12.9	10.4	-	12.8	29.5	15.0
6 days	3.7	-	6.3	-	11.5	-
8 days	6.8	-	-	-	12.4	-
CONTROL[b]	2.5±1.5	2.5±0.7	5.4±1.9	6.0±2.1	5.6±2.2	7.6±2.9

[a] Refers to the time α-methyltryptophan was given, before the radioisotope.
[b] Mean ± S.D. (7 animals)

Table VI. (cont'd)

TRYPTOPHAN-benzene-u-^{14}C

(C)	% ^{14}C recovered as $^{14}CO_2$ at:					
PRETREATMENT TIME[a]	2 hrs.		4 hrs.		6 hrs.	
	INTACT	ADREN.	INTACT	ADREN.	INTACT	ADREN.
0	-	5.5	-	-	-	11.4
1 hr.	6.9	-	-	-	12.6	-
3 hrs.	4.1	6.2	6.2	9.3	8.2	11.6
14 hrs.	13.2 3.9	8.2 -	18.9 6.6	12.7 -	21.2 6.1	15.4 -
1 day	13.3 -	5.4 6.2	19.4 -	9.3 -	22.8 -	11.6 9.9
3 days	11.1	4.8	15.7	8.4	17.6	11.7
5 days	4.4	-	6.5	-	8.0	
CONTROL[b]	5.8±1.4	4.6±1.3	6.9-	7.2±2.4	10.7±1.9	9.5±3.1

[a] Refers to the time α-methyltryptophan was given, before the radioisotope.
[b] Mean ± S.D. (7 animals)

at a later time than in tryptophan-treated animals. The maximum rate of oxidation of tryptophan-^{14}C was observed in animals pretreated with the amino acid analogue one day before the isotope was given as opposed to a zero pretreatment time with tryptophan. It is evident from these results that adrenalectomized animals respond to α-methyltryptophan, but to a lesser extent. The % ^{14}C recovered as $^{14}CO_2$ from tryptophan-3'-^{14}C (at 6 hrs.) in intact rats pretreated with the inducing agent at 14 hrs., 1 day, 3 days, were 15.7%, 18.8%, 16.2%, whereas in adrenalectomized rats the corresponding

values were 13.3%, 12.3%, 12.2%. It can be concluded that the prolonged induction of tryptophan pyrrolase by ∝-methyltryptophan is a result of two mechanisms, a substrate-type induction, concomitant with a hormonal induction mediated by the adrenal glands. These results were confirmed using T_2 and T_B.

b. Effect of DL-∝-Methyltryptophan on the Metabolism of Endogenous Tryptophan

Moran and Sourkes (168) have shown that the induction of tryptophan pyrrolase by ∝-methyltrytophan causes an increased breakdown of exogenous tryptophan. This has been verified in the previous section. The objective of this study was to determine whether the induction of tryptophan pyrrolase results in an increased breakdown of endogenous tryptophan. It would be more difficult to study this process using tryptophan as the inducing agent because of the problem of distinguishing endogenous from exogenous tryptophan.

Urinary levels of kynurenic acid and xanthurenic acid have been measured by several groups as an index of the rate of tryptophan metabolism (67, 83). The excretion of these quinaldic acids following a large dose of tryptophan is known to increase (67). These acids were measured in the urines of rats (120-150 g) receiving an intraperitoneal injection of ∝-methyltryptophan (1 mmole/kg). The urines were collected for 24 hours beginning at various times after

the injection, except in the case of the 12 hour experiment.

Fig. 14 illustrates the effects of the α-methyl amino acid on the urinary excretion of these metabolites. Both kynurenic and xanthurenic acid concentrations are elevated in the urine for 2 days. This is probably a consequence of increased breakdown of endogenous tryptophan due to pyrrolase induction by α-methyltryptophan. After 5 days both quinaldic acids dropped below control values in the urine. This may be brought about by a mild tryptophan deficiency developed during the course of the 5 days when the pyrrolase levels were high (169).

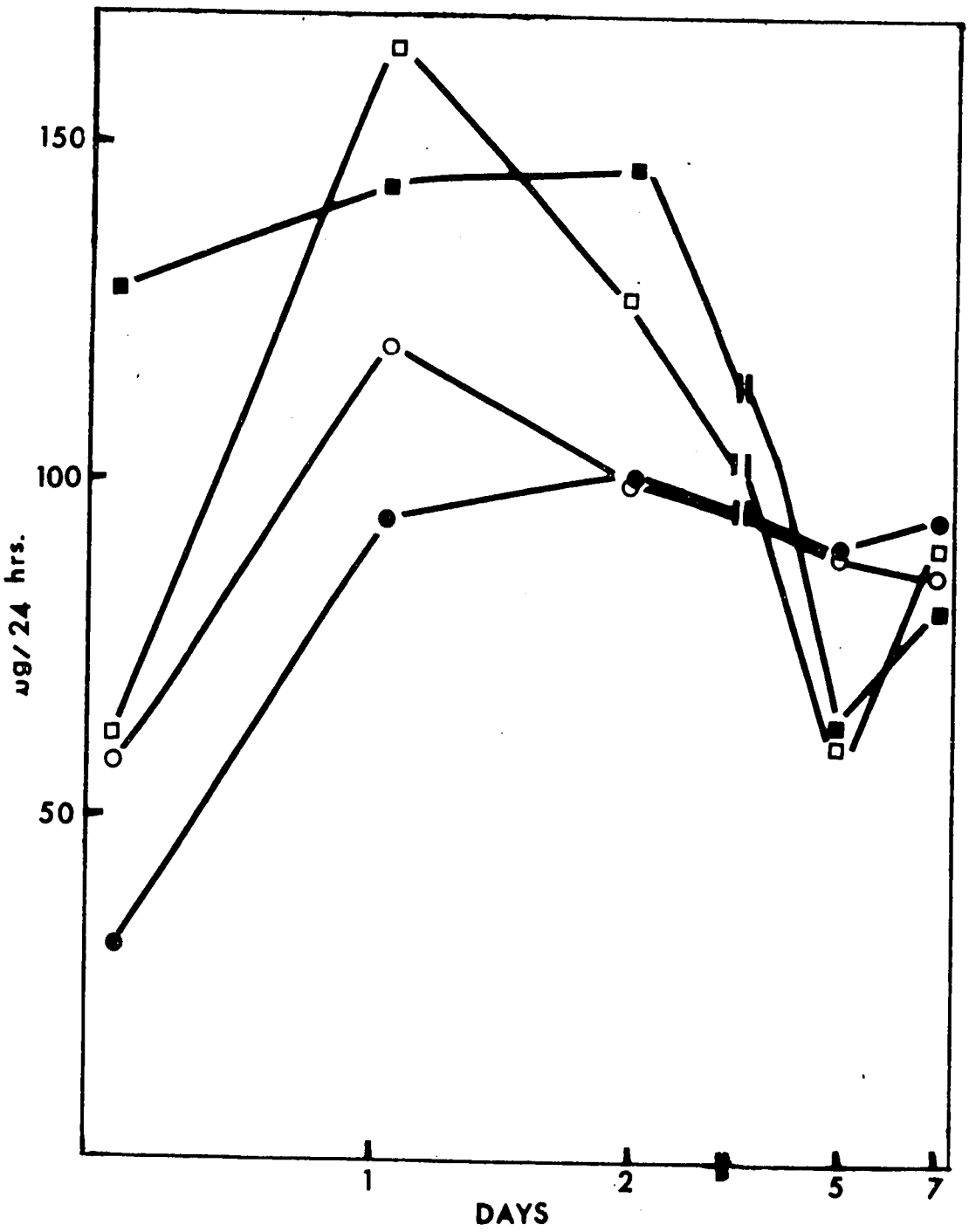


Fig. 14. The excretion of kynurenic and xanthurenic acids in urine following administration of DL- -methyltryptophan.H_2O.

Rats were injected with DL-α-methyltryptophan.H_2O (1 mmole/kg) and kynurenic (■-■), xanthurenic (□-□) acids were measured. Controls received 0.9% NaCl: kynurenic (●-●) and xanthurenic (○-○) acids. The abscissa is scaled logarithmically and denotes number of days after treatment. The first set of points denotes values for a 12 hour collection.

3. Effect of Metabolites Indigenous to the Rat on Tryptophan Metabolism

a. Effect of Tryptophan Metabolites on $^{14}CO_2$ Evolution from Tryptophan-^{14}C

When a tryptophan load (1 mmole/kg) is injected along with labeled tryptophan (10 μmole/kg), the excess metabolites formed may saturate enzymes in the degradative pathway resulting in lowered $^{14}CO_2$ production from the radioisotope. This process would modify the estimation of tryptophan pyrrolase activity *in vivo* as measured by this method.

Moran and Sourkes (168) have shown that a kynurenine load had no influence on the oxidation of tryptophan-3'-^{14}C, hence ruling out a significant isotope dilution effect. Yet there are still indications that excess metabolites of the degradative pathway may affect the generation of $^{14}CO_2$ from labeled tryptophan.

In a previous section it was demonstrated that the benzene-labeled tryptophan did not reflect the induction of tryptophan pyrrolase *in vivo* as distinctly as the other forms of radioactive tryptophan do, even though the control values for the radioisomers agreed well. A few possibilities were suggested to account for this observation including isotope dilution, product inhibition, side-product formation.

Recently several groups have given *in vitro* evidence for substrate or product inhibition along this pathway. Administered tryptophan inhibits 3-hydroxyanthranilic acid

oxygenase as measured in vitro (208). Wagner (144) showed that both 3-hydroxyanthranilic acid and 3-hydroxykynurenine inhibit tryptophan pyrrolase in vitro.

This experiment was performed: 1) to determine whether radioactive tryptophan is diluted by intermediates following a tryptophan load. The results of this experiment would be important in evaluating this method of measuring tryptophan pyrrolase activity in vivo; 2) to test whether this pathway of tryptophan is regulated by intermediates of the pathway.

Rats were injected intraperitoneally with the metabolite followed by one of the three radioisomers of tryptophan.

Kynurenine and 3-hydroxyanthranilic acid did not affect the oxidation of tryptophan-2-^{14}C. Kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid were without effect on the evolution of $^{14}CO_2$ from tryptophan-3'-^{14}C, Fig. 15.

If the levels of 3-hydroxyanthranilic acid in tissues were further raised by its administration along with tryptophan there was still no impaired oxidation of tryptophan-3'-^{14}C.

Kynurenine and 3-hydroxykynurenine did not alter the rate of $^{14}CO_2$ formation from the benzene-labeled tryptophan. But 3-hydroxyanthranilic acid did reduce the formation of respiratory $^{14}CO_2$ from the latter form of radioactive tryptophan (Table VII).

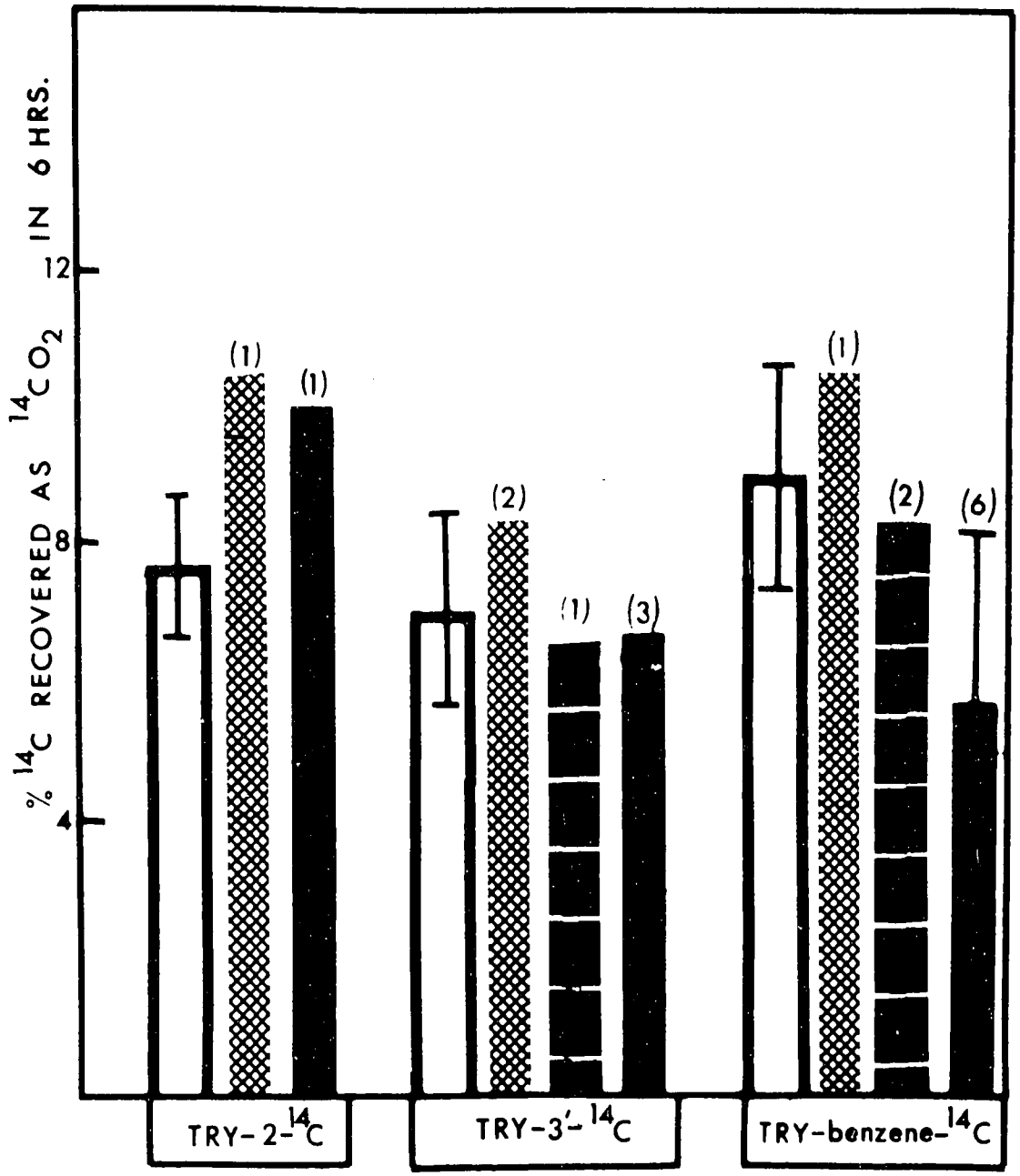


Fig. 15. The effect of tryptophan metabolites on the oxidation of DL-tryptophan-^{14}C *in vivo*.

Adrenalectomized rats were injected intraperitoneally with 0.9% NaCl □, kynurenine ▩, 3-hydroxykynurenine ▬, 3-hydroxyanthranilic acid ■ (all 1 mmole/kg). Immediately afterwards, the indicated isotope was given on the contralateral side of the abdomen (1.08 mc/mM, 0.14 mg). Number of experiments are indicated in parentheses.

Table VII. The effect of 3-hydroxyanthranilic acid on the oxidation of tryptophan-benzene-^{14}C *in vivo*.

Adrenalectomized rats were treated with 3-hydroxyanthranilic acid as indicated in the legend, Fig. 15. Results are expressed as % inhibition

$$(100\ \% - \frac{\%\ ^{14}C \text{ recovered as } ^{14}CO_2 \text{ - expt'l}}{\%\ ^{14}C \text{ recovered as } ^{14}CO_2 \text{ - control}} \times 100)$$

EXPT No:	% INHIBITION AT:		
	2 hrs.	4 hrs.	6 hrs.
1	96%	-	94%
2	57%	58%	59%
3	0	0	0
4	49%	46%	44%
5	56%	50%	47%
6	44%	36%	31%

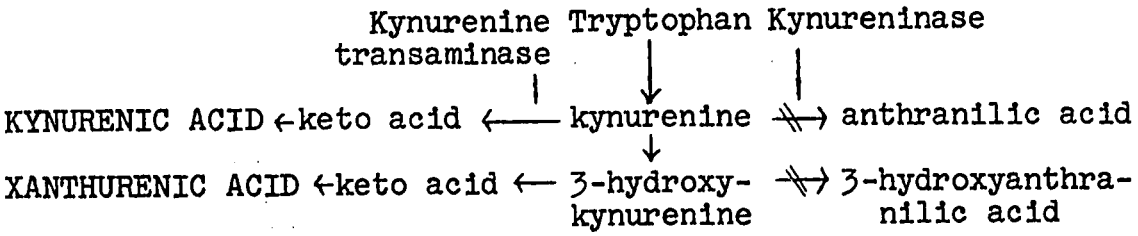
The values are quite variable nevertheless the inhibitory effect was present in 5 of 6 experiments.

These results indicate that there is no appreciable dilution of the metabolites of tryptophan-3'-^{14}C and tryptophan-benzene-^{14}C metabolism by the primary endogenous metabolites. Formation of $^{14}CO_2$ from benzene-labeled tryptophan was lowered following an injection of 3-hydroxyanthranilic acid. This may indicate a rate-limiting step in the pathway (by an in vivo method) or may reflect inhibition of 3-hydroxyanthranilic acid oxygenase by its substrate or products of the reaction.

There was no evidence for inhibition of tryptophan pyrrolase by the products of tryptophan metabolism which have been shown to do so by in vitro methods (144).

b. Effects of Vitamin B_6-Deficiency on Tryptophan Oxidation In Vivo

Several investigators have postulated that the elevated xanthurenic acid levels in vitamin B_6-deficiency are due to a shunting of 3-hydroxykynurenine into alternate pathways, because of the reduction of kynureninase -- a B_6-dependent enzyme -- during the deficiency (65-67, 69).



However, elevated xanthurenic acid levels were detected in the vitamin-deficient animals only after a tryptophan load was given. Under physiological conditions, the strongest evidence that there is a reduction of kynureninase activity has come from in vitro measurements of this enzyme (62, 63, 65). It is not established whether the reduction of activity of kynureninase is of physiological importance and results in a decreased formation of anthranilic acid and 3- hydroxy-anthranilic acid. The purpose of this study was to determine whether the pathway which is responsible for the formation of these metabolites is actually impaired in B_6-deficiency in vivo, under physiological conditions, i.e. without a load of tryptophan. This was done by measuring the rate of conversion of tryptophan-^{14}C to $^{14}CO_2$ in vitamin B_6-deficient and supplemented rats.

It is established that tryptophan pyrrolase is not a B_6-dependent enzyme (65). Therefore tryptophan-2-^{14}C served as a control for the other isomers of tryptophan. Theoretically, if kynureninase activity is lowered, it should be reflected in the oxidation of both tryptophan-3'-^{14}C and tryptophan-benzene-^{14}C because $^{14}CO_2$ is produced from these isomers beyond the level of kynureninase action.

Animals were placed on a vitamin B_6-deficient diet as described under "Methods". At the end of one month, 4 supplemented and 4 deficient rats were injected intraperitoneally with tryptophan-2-^{14}C, tryptophan-3'-^{14}C or tryptophan-benzene-

u-^{14}C. The fourth set was given a tryptophan load followed by tryptophan-3'-^{14}C.

The oxidation of tryptophan-2-$^{14}C \longrightarrow {}^{14}CO_2$ was not affected by the deficiency (Table VIII). On the other hand, the side-chain-and benzene-labeled tryptophans were oxidized to a lesser extent than corresponding controls. The degree of impairment, about 40%, was the same with both isomers. However, when a deficient (Set 4) animal was treated with excess tryptophan, the rate of oxidation of the isotope was reduced to 30% of control values (70% impairment). A week later another set (Set 5) was treated with tryptophan-benzene-^{14}C. Once again a reduction in the rate of oxidation of tryptophan was observed. The final sets (6, 7) were used two weeks later. The reduction in $^{14}CO_2$ formation from tryptophan-3'-^{14}C was evident but remained at the level attained 3 weeks previously. Alanine-^{14}C, the radioactive metabolite of the side-chain labeled tryptophan, was not oxidized as rapidly to $^{14}CO_2$ by the deficient animals; nevertheless the percent of control was at least 10% above the tryptophan-3'-^{14}C value, indicating that part of the impairment may be at the level of kynureninase.

These results demonstrate that there is a reduction in the rate of oxidation of tryptophan metabolites in vitamin B_6-deficient rats. This impairment is reflected in the amount of expired $^{14}CO_2$ from both tryptophan-3'-^{14}C and tryptophan-benzene-^{14}C. Part of the observed impairment may

Table VIII. The effect of a vitamin B_6-deficiency diet on the oxidation of DL-tryptophan-^{14}C in vivo.

Rats were made deficient as described under "Methods". After a period of time on the diet the animals were injected with equimolar amounts of each form of tryptophan-^{14}C (1.08 mc/mM) or alanine-u-^{14}C (3.0 mc/mM). The figures in parentheses denote % of control.

				% ^{14}C recovered as $^{14}CO_2$ at		
SET NO.	ISOMER	B_6 SUPPLE(+) DEF(-)	WEEKS ON DEF	2 hrs.	4 hrs.	6 hrs
1	TRYPTOPHAN-2-^{14}C	+ / -	4	1.2 / 1.2 (100%)	3.9 / 4.0 (102%)	5.3 / 5.5 (10
2	TRYPTOPHAN-3'-^{14}C	+ / -	4	4.3 / 2.4 (56%)	7.1 / 4.4 (62%)	8.1 / 5.1 (63
3	TRYPTOPHAN-b-u-^{14}C	+ / -	4	3.9 / 2.2 (56%)	6.1 / 3.7 (61%)	7.7 / 4.7 (61
4	TRYPTOPHAN-3-^{14}C[a]	+ / -	4	13.2 / 3.8 (29%)	36.4 / 10.0 (27%)	39.8 / 12.6 (32
5	TRYPTOPHAN-b-u-^{14}C	+ / -	5	7.1 / 4.5 (63%)	11.8 / 7.6 (65%)	13.3 / 8.5 (64
6	TRYPTOPHAN-3'-^{14}C	+ / -	7	8.1 / 5.9 (73%)	13.8 / 9.3 (67%)	13.9 / 10.0 (72
7	ALANINE-u-^{14}C	+ / -	7	62.7 / 46.2 (73%)	73.2 / 59.2 (81%)	75.2 / 64.5 (86

[a] rats were injected simultaneously with a tryptophan load (1 mmole/kg)

be accounted for by a decreased conversion of alanine to CO_2. This finding has recently been confirmed (209). Following a tryptophan load, the rate of metabolism of tryptophan-^{14}C was

30% of the control values, a result which lends support to the proposal that kynureninase activity is reduced *in vivo*, in B_6-deficiency, and that the enzyme is saturated readily. It is interesting to note that control values increased with increasing time on the diet. This may reflect an induction of tryptophan pyrrolase by the constituents of the diet.

c. Effect of Copper-Deficiency on Tryptophan Pyrrolase Activity in the Rat.

Recently Feigelson *et al.* have given evidence for a role of copper in bacterial tryptophan pyrrolase (128). The dependence of mammalian tryptophan pyrrolase on copper was studied by assaying the enzyme from livers of copper-deficient rats. Reports have shown that certain copper-dependent enzymes can be identified by this method (210,211).

The author is indebted to Mr. K. Lloyd for providing the copper-deficient animals and Mr. G. Gregoriadis for analyzing copper levels in the liver.

It is evident from the results (Table IX) that a reduction of liver copper levels does not lead to a lowering of tryptophan pyrrolase activity.

Copper deprivation prevents the normal absorption of iron from the gastrointestinal tract resulting in a reduction of total body iron (212). Although hematin is a cofactor of tryptophan pyrrolase, that fraction of the enzyme activity

Table IX. The activity of tryptophan pyrrolase in copper-deficient rats.

Rats were placed on a copper-deficient diet as described in "Methods". Tryptophan pyrrolase was assayed in the presence and absence of its co-factor, hematin. Results are expressed as μmoles kyn/gm/hr.

RAT NO.	CONTROL			LIVER Cu^{++}
	+HEMATIN	-HEMATIN	RATIO[a]	μg/g wet wt.
1	4.7	2.3	0.49	5.04
2	5.6	3.6	0.64	5.08
3	5.9	3.5	0.59	5.00
4	6.0	2.6	0.43	4.30
5	6.7	4.5	0.67	4.74
6	7.3	4.0	0.55	5.23
	6.0±0.9[b]	3.4±0.8[b]	0.56±0.09[b]	

RAT NO.	DEFICIENT			LIVER Cu^{++}
	+HEMATIN	-HEMATIN	RATIO[a]	μg/g wet wt.
1	6.5	3.8	0.59	1.74
2	8.6	3.9	0.45	2.13
3	6.1	2.8	0.46	2.03
4	6.4	2.5	0.39	2.24
5	6.7	3.4	0.51	2.11
6	5.6	3.0	0.54	2.64
	6.7±1.1[b]	3.2±0.6[b]	0.49±0.07[b]	

[a] Ratio: $\frac{\text{activity in absence of hematin}}{\text{activity in presence of hematin}}$

[b] Mean ± standard deviation

which represented the enzyme combined with endogenous co-factor was unchanged in copper-deficient animals. The ratio of endogenous holoenzyme to total enzyme was slightly but not significantly lowered. Therefore copper-deficiency does not appear to reduce the availability of iron for tryptophan pyrrolase activity.

These results are not conclusive evidence that tryptophan pyrrolase is not a copper-dependent enzyme. The enzyme may bind copper tenaciously so that the activity cannot be reduced by a deprivation of copper (128).

4. Effect of Drugs on Tryptophan Metabolism

a. Tryptophan Analogues

Although the pyrrolase pathway of tryptophan metabolism is implicated as the one regulating tissue concentrations of tryptophan, there are only a few drug studies on this metabolic route and most of these are based on experiments done *in vitro*. The present investigation employs the *in vivo* method described in previous sections (p. 59). The immediate objective of this study was to determine whether there are other inducing agents of tryptophan pyrrolase besides tryptophan and α-methyltryptophan. The results presented in this section will be compared with results obtained from *in vitro* studies (p.140).

Unless stated otherwise, adrenalectomized rats were used to eliminate the possibility of stress-induction of tryptophan pyrrolase. It is known that many drugs can do this in intact animals (10, 157).

Tryptophan analogues (Table X, Section A) were injected at the dose level usually used to achieve high tryptophan pyrrolase levels with tryptophan. The hydroxytryptophan and tryptamine analogues were given at lower doses, because of their pharmacological effects.

Of the compounds tested, only the D-isomer of tryptophan had an appreciable inductive effect *in vivo*. Animals injected with D-tryptophan oxidized about 17% of the radioactive tryptophan to $^{14}CO_2$ within 6 hours. This is in con-

Table X. The effect of indoles on the rate of oxidation of DL-tryptophan-$^{14}C \longrightarrow {}^{14}CO_2$.

Adrenalectomized rats were injected intraperitoneally with the drug and tryptophan-3'-^{14}C (1.08 mc/mmole) was given on the contralateral side. Results are expressed as % of control:

$$\left(= \frac{\%\ ^{14}C \text{ recovered as } {}^{14}CO_2 \text{- expt'l}}{\%\ ^{14}C \text{ recovered as } {}^{14}CO_2 \text{- control}} \times 100 \right)$$

SECTION A - TRYPTOPHAN DERIVATIVES

DRUG	DOSE mg/kg	% OF CONTROL AT: 2 hrs.	6 hrs.
α-Hydrazinotryptophan	50	58%	47%
Hypaphorine	50	76%	72%
"	200	25%	26%
5-Methyltryptophan	200	100%	74%
6-Methyltryptophan	200	55%	52%
D-Tryptophan	200	315%	313%
"	200	407%	316%
Tryptophol	50	78%	70%

SECTION B - HYDROXYTRYPTOPHAN DERIVATIVES

α-Methyl-5-hydroxytry	150	110%	134%
α-Hydrazino-5-hydroxytry	50	48%	48%
5-Hydroxytryptophan	50	100%	100%
4-Hydroxytryptophan	50	76%	83%

trast to the increased oxidation of DL-tryptophan-^{14}C following injection of L-tryptophan-^{12}C, 37%. A comparison of the effects of D- and L-tryptophan-^{12}C on the oxidation of exogenous tryptophan is given in Fig. 16. It has been shown (Table II) that an appreciable amount of the D-tryptophan is metabolized within 6 hours of administration. Therefore the inductive process may be subsequent to the inversion of D-tryptophan to the L- form. α-Methyl-5-hydroxytryptophan caused an accelerated breakdown of tryptophan-3'-^{14}C. When this experiment was repeated, with tryptophan-2- and -3'-^{14}C, the $^{14}CO_2$ production was increased 15% above control values. This degree of acceleration is quite small in comparison with the effects of tryptophan (600% above control values) or α-methyltryptophan (300% above control values).

At lower doses (50-100 mg/kg) α-methyl-5-hydroxytryptophan seemed to exert an inhibitory effect. Hypaphorine, the betaine of tryptophan, reduced the rate of oxidation of tryptophan by 75%, at a dose level of 200 mg/kg. The 6-methyl analogue halved tryptophan oxidation at this dose.

α -Hydrazinotryptophan was the most effective inhibitor of tryptophan metabolism (40% -50%, 50 mg/kg). Of the 5-hydroxytryptophan analogues (Section B), only α-hydrazino-5-hydroxytryptophan exerted a consistent inhibitory effect. Several experiments with tryptophol yielded inconsistent results.

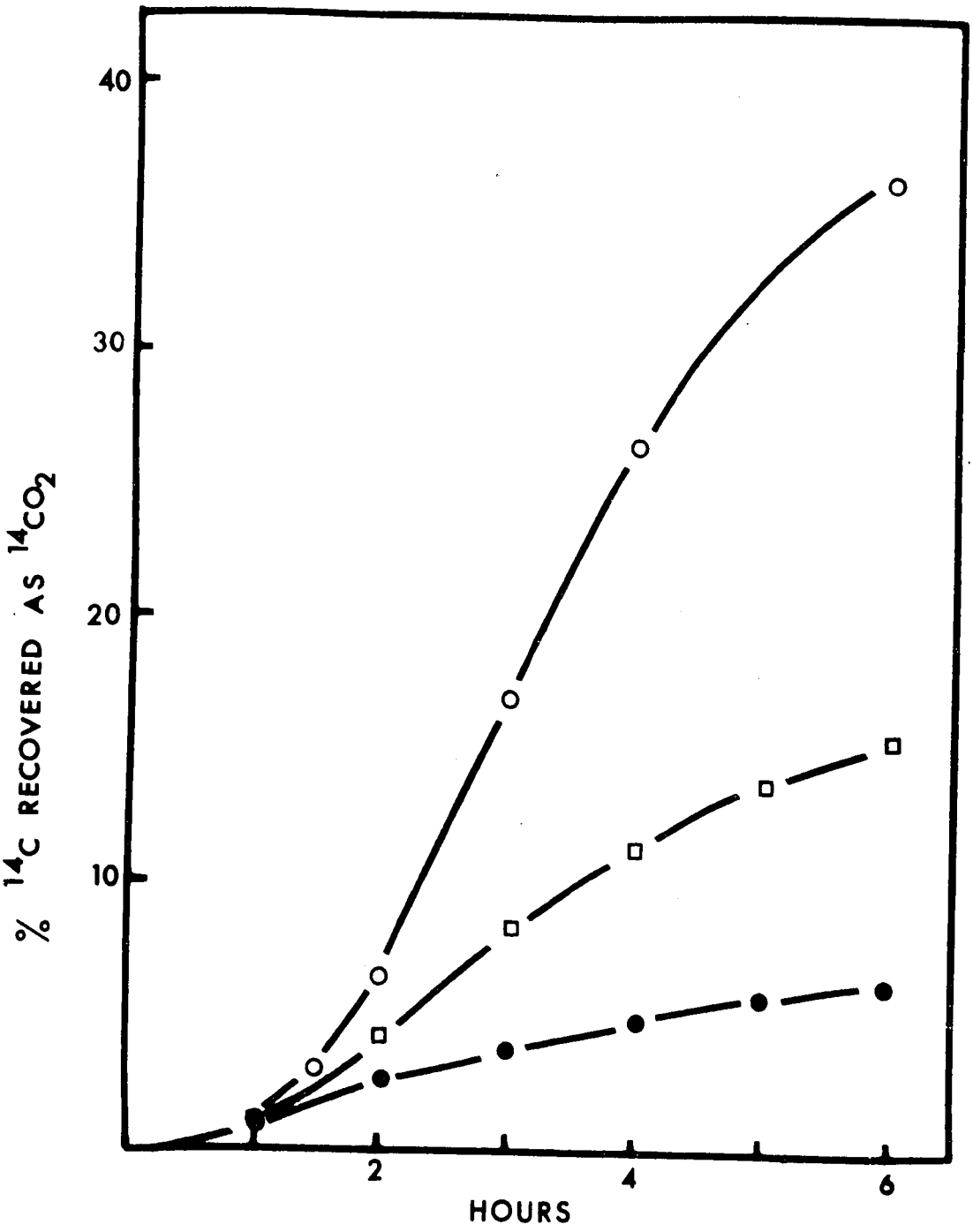


Fig. 16. The effect of L-tryptophan and D-tryptophan on the induction of tryptophan pyrrolase as measured by the rate of oxidation of DL-tryptophan-3'-^{14}C $\longrightarrow$ $^{14}CO_2$.

Rats were injected intraperitoneally with 1 mmole of L-tryptophan (○ - ○) or D-tryptophan (□-□) followed by DL-tryptophan-3'-^{14}C (1.08 mc/mM) on the contralateral side of the abdomen. Controls received 0.9% NaCl, (●-●).

These inconsistencies may be due to variations in the metabolism of this substance.

The tryptamine derivatives (Table XI) especially N,N-dimethyltryptamine and N-acetyltryptamine, were potent inhibitors of tryptophan oxidation in vivo.

Table XI. The effect of tryptamine derivatives on the oxidation of tryptophan-3'-^{14}C.

See Table X for description.

DRUG	DOSE mg/kg	% OF CONTROL AT: 2 hrs.	6 hrs.
Serotonin[a]	25	110%	110%
Tryptamine	50	113%	89%
α-Methyltryptamine	50	93%	85%
α-Ethyltryptamine	50	63%	80%
N,N-Dimethyltryptamine	50	46%	52%
N,N-Diethyltryptamine	50	61%	89%
N-Acetyltryptamine	50	26%	41%

[a]Given tryptophan-2-^{14}C

It can be concluded from these results that the current concept of a very specific substrate induction of tryptophan pyrrolase remains unchallenged; no other inducers of the enzyme have been identified with the exception of D-tryptophan already established as an inducing agent by measurement of

the enzyme *in vitro* (140). Tryptophan oxidation *in vivo* is inhibited by a variety of indoles, the most potent being hydrazines and substituted amines: hydrazino analogues of tryptophan and tryptamine derivatives. Interestingly, the indoles indigenous to the rat, serotonin, tryptamine, and 5-hydroxytryptophan, had no effect *in vivo*.

Conclusive structure-activity relationships were not observable because the pharmacological properties of some of these compounds prohibited injection at comparable doses.

Although it is tempting to conclude that these indoles act as antimetabolites of tryptophan, this cannot be asserted. Additional experiments were performed in an effort to localize further the site of action of the compounds, including studies *in vitro* (Sec. 5).

α-Hydrazinotryptophan was injected into rats, along with each of the three radioisomers of tryptophan. This was done to ascertain whether the inhibitory effect could be detected equally with all three forms of radioactive tryptophan. It is evident from Fig. 17 that the compound inhibited the oxidation of both tryptophan-2- and 3'-^{14}C but had little effect on the benzene-labeled tryptophan.

If this analogue were acting solely on the tryptophan transport system, the transport and consequently the oxidation of all three isomers would be reduced.

A more careful examination of the results (Table XII)

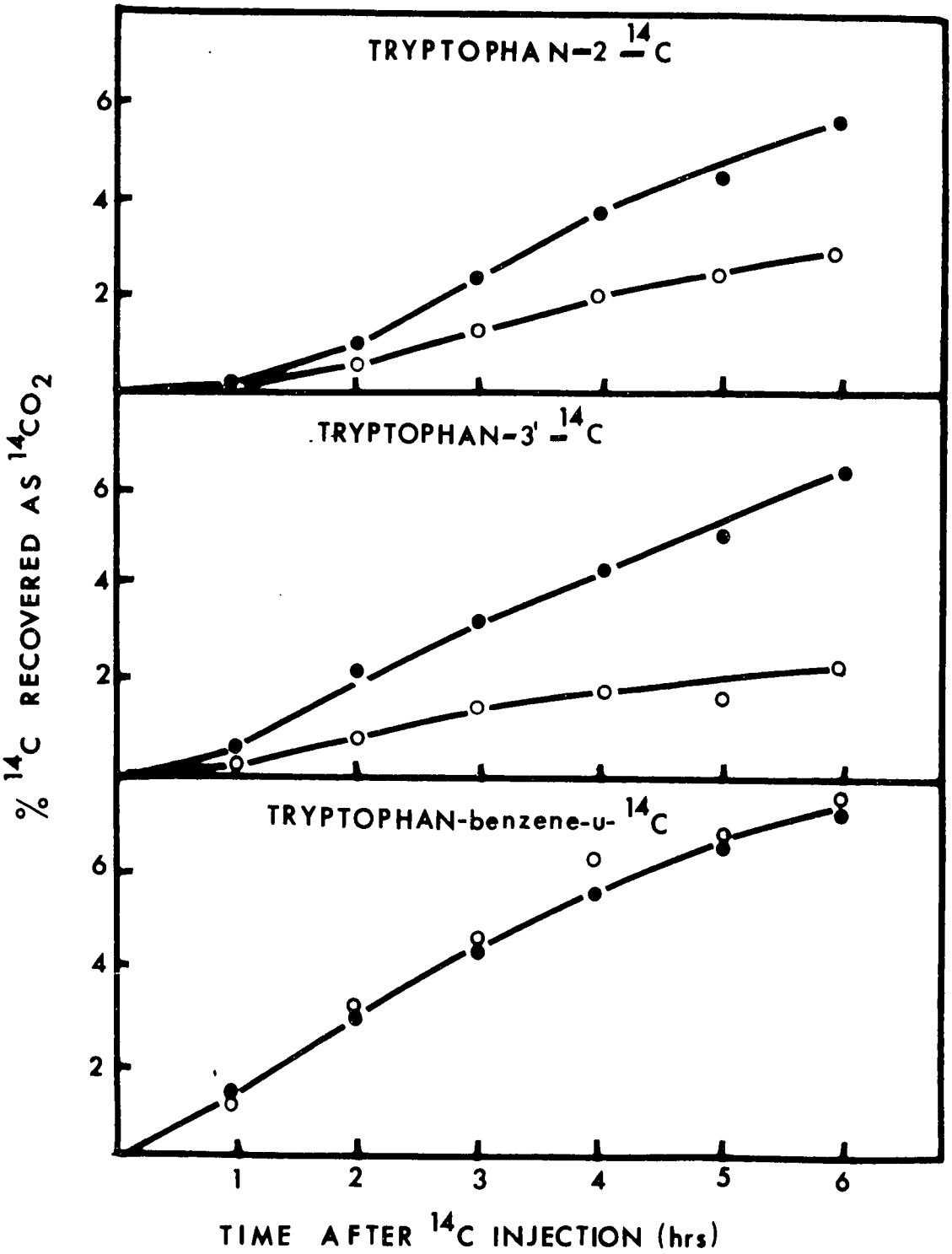


Fig. 17. The effect of α-hydrazino tryptophan on the rate of oxidation of DL-tryptophan labeled with ^{14}C in different positions.

α-Hydrazinotryptophan, (40-60 mg/kg) o - o, was injected intraperitoneally into adrenalectomized rats and DL-tryptophan-^{14}C (1.08 mc/mM) was given on the contralateral side of the abdomen. Controls (● - ●) received 0.9% NaCl.

Table XII. The effect of α-hydrazinotryptophan on the oxidation of DL-tryptophan labeled with ^{14}C in different positions. Please see Fig. 17 for doses. Control values are expressed as mean ± S.E. (4 animals).

ISOMER	% ^{14}C recovered as $^{14}CO_2$ at:		
	2 hrs.	4 hrs.	6 hrs.
TRYPTOPHAN-2-^{14}C	1.0	2.5	3.8
	0.6	2.4	3.0
	0.6	2.2	3.4
	0.5	1.8	2.8
CONTROL (mean± S.E.)	1.0±0.07	3.9±0.3	5.7±0.2
TRYPTOPHAN-3'-^{14}C	0.2	0.7	1.5
	-	1.4	2.1
	0.4	1.0	1.8
	1.5	2.7	3.8
CONTROL (mean± S.E.)	2.3 -	4.4±0.3	6.6±0.3
TRYPTOPHAN-benzene-u-^{14}C	3.4	-	8.5
	2.6	4.9	6.1
	3.4	7.4	8.5
	3.3	7.0	-
CONTROL (mean± S.E.)	2.9±0.1	5.6±0.1	7.2±0.1

reveals that the conversion of tryptophan-3'-^{14}C $\longrightarrow$ $^{14}CO_2$ is reduced (4 hrs. and 6 hrs.) to a greater extent than the pyrrole-labeled material. The % inhibition of latter is 44% at 6 hrs., whereas the oxidation of the side-chain labeled tryptophan was lowered by 65%.

This may indicate that the inhibitor has more than one site of action - either on the tryptophan degrading system or on alanine oxidation. Formate-^{14}C and alanine-^{14}C were injected into rats along with α-hydrazinotryptophan. Formate-^{14}C oxidation was lowered 12-18% by the injection of the indole. Alanine oxidation was reduced by 15%. The lack of effect on benzene ring-labeled tryptophan will be dealt with in the discussion.

α-Hydrazino-5-hydroxytryptophan was also tested further and found to inhibit both tryptophan-2- and -3'-^{14}C oxidation. The % inhibitions at 6 hrs. were 37% and 52% respectively, once again suggesting that this hydrazino derivative may have more than one site of action. α-Hydrazinotryptophan did not interfere with the appearance of radioactivity in the serum 15 minutes after administration of tryptophan-3'-^{14}C when both were given at the same time. One hour later the radioactivity in the serum was triple that of controls, (Table XIII).

The normal levels of radioactivity in the serum following α-hydrazinotryptophan would suggest that tryptophan transport was unaffected by the analogue. The increase at one hour may indicate that tryptophan metabolism is reduced, resulting in more circulating tryptophan.

Table XIII. The effect of ∝-hydrazinotryptophan on the radioactivity of serum following administration of DL-tryptophan-3'-^{14}C.

Rats were injected concurrently with ∝-hydrazino tryptophan (50 mg/kg) and DL-tryptophan-3'-^{14}C (4.7 mc/mM) on opposite sides of the abdomen. Blood was removed from the tail vein at 15, 60 minutes and analyzed for radioactivity. Results are an average of 2 animals.

TIME AFTER ^{14}C	cpm/ml serum	
	CONTROL	EXPT'L
15 min.	20,240	22,940
60 min.	8,120	25,480

b. Effect of Indole Alkaloids on Tryptophan Oxidation In Vivo

Several indole alkaloids are recognized as antimetabolites of serotonin, including yohimbine (176) and lysergic acid diethylamide (L.S.D.) (175). The purpose of this study was to learn whether the postulated antimetabolite activity attributable to the indole nucleus of a drug extends to antagonism toward the enzymes of tryptophan catabolism.

Representatives from four groups of indole alkaloids, the ergot, harmala, iboga and yohimbe families were used in this experiment. Animals were treated in the same manner as those injected with tryptophan analogues (Sec. 4a). The dosages were adjusted according to the pharmacological properties of the compounds. The results are shown in Table XIV.

Table XIV. The effect of indole alkaloids on DL-tryptophan-^{14}C oxidation in vivo.

Adrenalectomized rats were injected intraperitoneally with the alkaloid and DL-tryptophan-^{14}C (1.08 mc/mM, 0.8 μc) was given on the contralateral side of the abdomen. Results are expressed as % of control:

$$\left(\frac{\%\ ^{14}C \text{ recovered as } ^{14}CO_2\text{-expt'l}}{\%\ ^{14}C \text{ recovered as } ^{14}CO_2\text{-control}} \times 100\right)$$

ALKALOID	DOSE mg/kg	ISOMER T_2[a] or T_3[b]	% OF CONTROL AT: 2 hrs.	6 hrs.
ERGOTAMINE	4	T_2	141	119
"	5	T_2	38, 31	47, 19
"	5	T_3	119	103
DIHYDROERGOTAMINE	9	T_3	100	95
L.S.D.[c]	0.9	T_2	124	123
L.S.D.	0.9	T_3	105	155
HARMALINE	40	T_2	73	66
"	40	T_3	39	62
HARMALOL	40	T_3	59	75
IBOGAINE	20	T_2	89	95
"	40	T_3	79	90
IBOGAMINE	40	T_2	110	112
NORIBOGAINE	20	T_2	110	104
"	40	T_2	21	29
"	40	T_3	14	31
"	50	T_3	31	41
YOHIMBINE	5	T_2	54	50
"	5	T_3	58	69
CORYNANTHINE	100	T_2	35	37
"	100	T_3	66	69

[a] T_2 - tryptophan-2-^{14}C

[b] T_3 - tryptophan-3'-^{14}C

[c] L.S.D. - lysergic acid diethylamide

Ergotamine yielded inconsistent results; in two of four experiments the alkaloid caused a slight acceleration of tryptophan metabolism, in the rest it acted as a potent inhibitor. Dihydroergotamine was ineffective at double the dose of ergotamine. L.S.D. accelerated the formation of $^{14}CO_2$ from tryptophan slightly, perhaps as a result of its temperature-elevating effect. Harmaline, and its demethylated product, harmalol, suppressed the oxidation of tryptophan by 25% -38%, at 6 hrs. Further studies with harmaline are described below. Both ibogaine and ibogamine were weak inhibitors; another alkaloid of this group, noribogaine, (40-50 mg/kg) reduced tryptophan oxidation by 60% -70% during the 6 hours after its administration. At lower doses (20 mg/kg) it was ineffective. Yohimbine interfered with tryptophan oxidation, 30% -50% (5 mg/kg) whereas its geometric isomer, corynanthine, achieved a comparable effect only at 20 times this dose. The results indicate that the presence of the indole structure in a compound may well render it an antimetabolite of tryptophan; but this is not a necessary consequence, for further structural requirements must be fulfilled in many cases. One cannot conclude, on the basis of this type of experiment, that the inhibitors act directly on the tryptophan-degrading enzymes. Some of the alkaloids, are known to elicit blood pressure changes which may interfere with the transport of tryptophan. Further experiments were performed to lend support to or negate the

possibility of a specific action on tryptophan degradation. Harmaline and yohimbine were given with each of the radioisomers of tryptophan. Harmaline (Table XV) consistently reduced tryptophan-3'-^{14}C metabolism, the degree of inhibition being 50-60% within 4 hours and declining within 6 hours. As tryptophan-2-^{14}C was affected to a lesser extent than the side-chain labeled material, the possibility that harmaline may affect alanine oxidation was considered. At 38 mg/kg of harmaline, the rate of evolution of $^{14}CO_2$ from alanine-^{14}C was 91% of control values; at 50 mg/kg, alanine oxidation was reduced by 83%, indicating that harmaline has alternate sites of action. As with the hydrazino analogue of tryptophan, the metabolism of benzene-labeled tryptophan was unaffected.

Yohimbine (Table XVI) lowered $^{14}CO_2$ production from tryptophan-2-^{14}C, and possibly tryptophan-3'-^{14}C but not from the benzene-labeled tryptophan. Both harmaline and yohimbine had little or no influence on formate oxidation (Table XVII).

Table XVII. The effect of harmaline and yohimbine on formate oxidation.

Adrenalectomized rats were injected intraperitoneally with harmaline (50 mg/kg) or yohimbine (5 mg/kg) followed by Na formate-^{14}C (10 mc/mM). Results are expressed as % of control:

$$\left(\frac{\%\ ^{14}C \text{ recovered as } ^{14}CO_2\text{-expt'l}}{\%\ ^{14}C \text{ recovered as } ^{14}CO_2\text{-control}} \times 100\right)$$

	% OF CONTROL AT:		
	2 hrs.	4 hrs.	6 hrs.
HARMALINE	98%	100%	100%
YOHIMBINE	92	93	92

TABLE XV. The effect of harmaline on the rate of oxidation of DL-tryptophan, labeled with ^{14}C in different positions.

Rats were treated with harmaline (40mg/kg) as described in Table XIV.

ISOMER	% INHIBITION AT:		
	2 hrs.	4 hrs.	6 hrs.
TRYPTOPHAN-2-^{14}C	56%	43%	13%
	27%	34%	27%
	20%	20%	8%
TRYPTOPHAN-3'-^{14}C	62%	52%	62%
	56%	-	24%
	47%	23%	25%
	63%	59%	54%
TRYPTOPHAN-benzene-u-^{14}C	23%	27%	0
	41%	-	13%
	18%	10%	11%

Table XVI. The effect of yohimbine on the oxidation of DL-tryptophan labeled with ^{14}C in different positions.

Rats were treated as described in Table XIV, the dose of yohimbine being 50 mg/kg. Controls are expressed as mean ± S.E. (4 animals).

ISOMER	% ^{14}C recovered as $^{14}CO_2$ at:		
	2 hrs.	4 hrs.	6 hrs.
TRYPTOPHAN-2-^{14}C	0.6	1.7	2.8
	0.7	2.5	3.8
	0.1	0.3	1.0
	0.7	1.9	3.2
CONTROL (mean± S.E.)	1.3±0.05	3.6±0.05	6.0±0.1
TRYPTOPHAN-3'-^{14}C	3.4	5.9	7.3
	1.2	2.7	4.1
	1.8	4.5	4.2
	1.3	3.6	6.0
CONTROL (mean ± S.E.)	2.8±0.2	6.0±0.5	7.6±0.5
TRYPTOPHAN-benzene-u-^{14}C	2.2	-	6.3
	2.1	-	7.3
	2.6	-	6.8
	2.9	5.6	7.6
CONTROL (mean ± S.E.)	3.7±0.4	5.6 -	8.1±0.5

Tryptophan pyrrolase from yohimbine-treated rats was assayed and found to have the same activity as the controls, and the same degree of saturation with cofactor. This does not rule out the possibility that yohimbine inhibits the enzyme *in vivo*.

Radioactivity in liver and brain was increased above control values following administration of these alkaloids (Table XVIII). This would lend further support to the possibility that both alkaloids interfered with tryptophan degradation, not tryptophan transport.

Table XVIII. The radioactivity in liver and brain following administration of DL-tryptophan-2-^{14}C with harmaline or yohimbine.

Rats were treated as described in Table XIV. Tissue radioactivity was measured as described under "Methods". Results were calculated as cpm/mg wet wt. and are compared to control values.

		% OF CONTROL	
DRUG	% INHIBITION OF TRYPTOPHAN OXIDATION *IN VIVO* at 6 hrs.	$\left(\frac{\text{cpm/mg wet wt.-Expt'l}}{\text{cpm/mg wet wt.-Control}} \times 100\right)$	
		LIVER	BRAIN
HARMALINE	66%	135%	144%
YOHIMBINE	50%	139%	167%

The increased radioactivity could stem from high levels of a number of compounds derived from the administered tryptophan. Definite conclusions about the site of enzymic blockade by the

drugs cannot be reached until these metabolites are characterized. As tryptophan-2-^{14}C was used the metabolites to be sought would be tryptophan itself, N-formylkynurenine, 5-hydroxyindole derivatives and others in which the indole nucleus remains intact, as well as formate derivatives, which would carry the label.

c. Effect of Tyrosine Derivatives on Tryptophan Metabolism

Tryptophan and some of its derivatives are known to alter tyrosine metabolism (11). It was therefore considered of interest to determine whether tyrosine derivatives exert a reciprocal effect on tryptophan metabolism. Of the 6 derivatives, generically related to tyrosine, that were studied *in vivo* (Table XIX) only α-methyl- α-hydrazino-3,4-dihydroxyphenylpropionic acid (MK-485) and α-methyl-m-tyrosine retarded the degradation of tryptophan. Both of these compounds contain a free m-hydroxy group. α-Methyldopa, which also contains a m-hydroxy group, was ineffective. Other compounds that were devoid of inhibitory effects included α-methyltyrosine, α-methyl-m-methoxyphenylalanine, and α-methyl-3,4-dimethoxyphenylalanine.

It is interesting to note that the hydrazino analogue of α-methyldopa (MK-485) inhibited the oxidation of tryptophan-3'-^{14}C to a greater extent than that of tryptophan-2-^{14}C. These results are in agreement with the effects of the other hydrazino compounds studied (α-hydrazinotryptophan, α-hydrazino-5-hydroxytryptophan).

Table XIX. The effect of aromatic ∝-methylamino acids on DL-tryptophan metabolism.

The compounds were injected intraperitoneally into adrenalectomized rats and DL-tryptophan-3'-^{14}C was given at the same time (1.08 mc/mM). Controls received 0.9% NaCl.

DRUG	DOSE mg/kg	% OF CONTROL AT: 2 hrs.	4 hrs.	6 hrs.
∝-Methyl-∝-hydrazino-3,4-dihydroxyphenylpropionic acid	110[a]	80	98	100
	200[a]	50	59	64
	200	28	33	42
∝-Methyl-m-methoxyphenylalanine	200[a]	87	88	87
	200	132	143	143
L-∝-methyldopa	200	80, 91	95	98
∝-methyl-3,4-dimethoxyphenylalanine	200	126	124	120
∝-Methyl-m-tyrosine	180	85	84	80
∝-Methyl-tyrosine	200	88	90	96

[a] Given tryptophan-2-^{14}C

d. Effect of Adrenergic Agents on Tryptophan Metabolism In Vivo.

In a previous section (4b), yohimbine was shown to inhibit tryptophan oxidation in vivo. Among the known pharmacological actions of this alkaloid is its adrenergic blocking effect (186). Although Knox ruled out the possibility of an adrenergic stimulation of tryptophan pyrrolase activity, recent reports have caused renewed interest in this mechanism. Chytil (132, 133) and others (122, 134) have shown that cyclic -AMP (cyclic-adenosine-3', 5'-monophosphate), one of the mediators of the adrenaline response, reactivates inactivated tryptophan pyrrolase in vitro. In view of these findings, and the effect of yohimbine, it was decided to reevaluate the role of adrenergic mechanisms in relation to tryptophan degradation in vivo. Adrenalectomized rats were used throughout to prevent a stress-induced response.

i. Effects of adrenergic agents:

In the dose range of 0.25-0.75 mg/kg, adrenaline impaired the formation of $^{14}CO_2$ from tryptophan-2-^{14}C, (Table XX). At a higher dose (1.0 mg/kg) half the animals tested expired more $^{14}CO_2$ than controls; the others did not. The increased output of $^{14}CO_2$, when it occurred, was very low (10-60%) in comparison to the response obtained with tryptophan (500-700%). Moreover, the maximum increase above control values occurred within

Table XX. The effect of L-adrenaline, L-noradrenaline and DL-isoproterenol on DL-tryptophan oxidation in vivo.

Adrenalectomized rats were injected intraperitoneally with the catecholamine followed by DL-tryptophan-2-^{14}C (1.08 mc/mM) on the contralateral side of the abdomen. Results are expressed as % of control:

$$\left(\frac{\%\ ^{14}C \text{ recovered as } ^{14}CO_2 \text{ - expt'l}}{\%\ ^{14}C \text{ recovered as } ^{14}CO_2 \text{ - control}} \times 100\right)$$

DRUG	DOSE mg/kg	% OF CONTROL AT: 2 hrs.	4 hrs.	6 hrs.
ADRENALINE	.25	80	73	70
	.50	71	86	89
	.75	97	99	95
	1.0	290	94	-
	1.0	64	81	84
	1.0	145	125	111
	1.0	160	160	148
	1.0	156	124	116
	1.0	69	-	-
	1.0	25	28	-
	0.5[a]	75	82	86
	1.0[a]	50	51	54
NORADRENALINE	.25	120	120	119
	.50	97	104	98
	1.0	78	97	98
ISOPROTERENOL	.05	90	91	95
	.50	57	70	74
	1.0	56	63	67
DOPAMINE	1.5	110	110	96

[a] Given tryptophan-3'-^{14}C

2 hours of injection, and declined thereafter, whereas the tryptophan-response reached a peak at 6 hours or more. This would suggest a mechanism unrelated to protein synthesis or protein turnover. The survival of animals treated with this amount of adrenaline was very low. Noradrenaline and dopamine exerted no effect whereas isoproterenol reduced tryptophan oxidation in the dose range of 0.5-1.0 mg/kg.

In order to ascertain whether adrenaline was acting non-specifically, e.g. on respiration or on the oxidation of formate, ^{14}C-Na formate was given along with adrenaline. The rate of oxidation of formate was lowered by 29%, 15%, 9%, at 2, 4, 6 hours (resp.) following administration of adrenaline. This would suggest that the acceleration of $^{14}CO_2$ formation from tryptophan-2-^{14}C was not due to increased ventilation. The reduction of formate metabolism by adrenaline may account for some of the inconsistencies observed.

Adrenaline (1 mg/kg) did not increase tryptophan pyrrolase activity as measured *in vitro* (Table XXI).

Table XXI. The effect of L-adrenaline on tryptophan pyrrolase activity.

Rats were injected with 1 mg/kg L-adrenaline. One hour later, the animals were killed and tryptophan pyrrolase was assayed in the liver, as described under "Methods". Controls received 0.9% NaCl.

	µmoles kyn/gm/hr		
TREATMENT	TOTAL ENZYME	ENDOGEN. HOLOENZYME	30' INACT.
Control	2.7	1.6	1.8
Control	3.0	1.0	1.1
Adrenaline	2.2	1.2	1.4
Adrenaline	2.1	1.1	0.8

The degree of inactivation of the enzyme from adrenaline-treated animals was unaltered, suggesting that either tissue cyclic-AMP was not elevated following adrenaline or that, if elevated, this endogenous nucleotide has no effect on tryptophan pyrrolase.

ii. Effects of adrenergic blocking agents:

Because of the contradictory results with adrenaline and the difficulty in maintaining the animals after administration of adrenaline, another approach to this problem was attempted. Adrenergic blocking agents were injected into adrenalectomized rats and their influence on the metabolism of tryptophan was studied. The effects of yohimbine, ergotamine, and dihydroergotamine, all α-blockers have already been discussed (Section 4b). The α-blockers, dibenzyline, phentolamine and the β-blockers, pronethalol, propranolol and dichloroisoproterenol (DCI) all retarded the formation of $^{14}CO_2$ from pyrrole-labeled tryptophan (Fig. 18). Of the two α-blockers mentioned, dibenzyline was the more effective, and of the β-blockers, propranolol was the weakest, the other two having an equivalent response. The level of inhibition of oxidation of tryptophan-2- or -3'-^{14}C established by dibenzyline at 2 hours remained unaltered for at least 6 hours (Table XXII) and pretreatment at 1 hour did not diminish the response.

Phentolamine was a weak inhibitor and dibenamine was ineffective. If given 1 hour prior to tryptophan, phentolamine

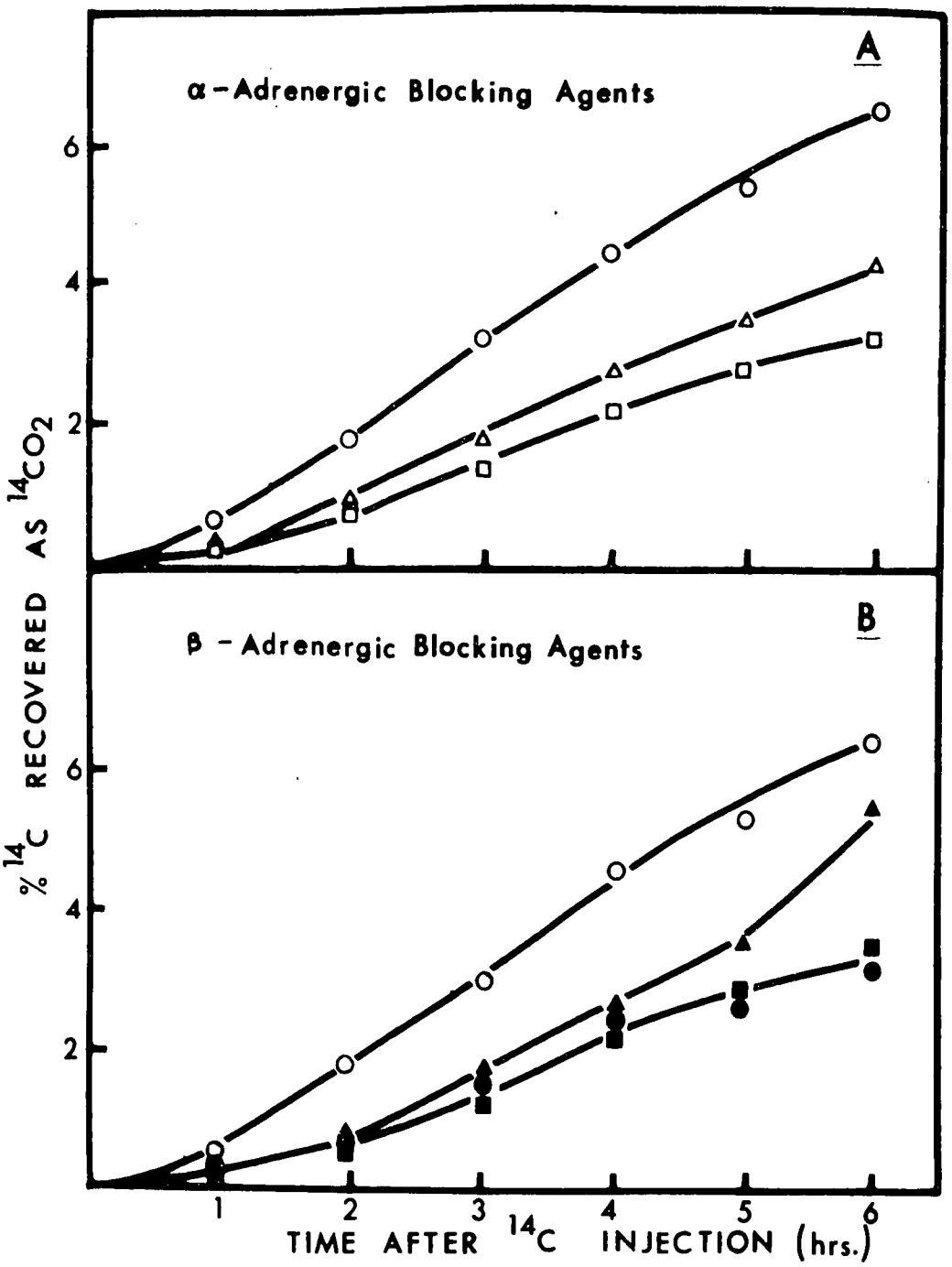


Fig. 18. The effect of α- and β-adrenergic blocking agents on DL-tryptophan-2-^{14}C oxidation in vivo.

Animals were treated as described in Table XXII.
A Control (○ - ○), dibenzyline (□ - □), phentolamine (△ - △).
B Control (○ - ○), pronethalol (■ - ■), propranolol (▲ - ▲), dichloroisoproterenol (● - ●).
Each point is an average of at least 3 animals.

Table XXII. The effect of α-adrenergic blocking drugs on DL-tryptophan oxidation in vivo.

Adrenalectomized rats were injected intraperitoneally with the blocking agent followed by DL-tryptophan-2-^{14}C (1.08 mc/mM) on the contralateral side. Controls received 0.9% NaCl. Results are expressed as % inhibition with respect to individual controls.

DRUG	DOSE mg/kg	% INHIBITION AT: 2 hrs.	6 hrs.
DIBENZYLINE	10	67	65
	10	0	9.0
	10	59	58
	10[a]	48	48
	10[b]	62	54
DIBENAMINE	10[a]	0	11
	30	27	2.0
PHENTOLAMINE	10	0	0
	20	0	19
	20	27	21
	20	21	27
	20[a]	69	70
	20[b]	34	17
	20[b]	71	60

[a] Tryptophan-3'-^{14}C injected instead of tryptophan-2-^{14}C

[b] Drug injection 1 hour before radioisotope

Table XXIII. The effect of β-adrenergic blocking drugs on DL-tryptophan oxidation in vivo.

Animals were treated as described in Table XXII.

DRUG	DOSE mg/kg	% INHIBITION AT: 2 hrs.	6 hrs.
PRONETHALOL	30	56	23
	30	54	58
	30	36	7.0
	30	90	71
	30[a]	33	22
	30[b]	68	35
PROPRANOLOL	30	50	38
	30	50	27
	30[a]	7	0
	30[b]	46	6
	40[b]	63	22
DICHLOROISOPRO-TERENOL	30	44	54
	30	73	54
	30	0	0
	30	50	51
	30b	71	36
	30[a, b]	56	42

[a] Tryptophan-3'-^{14}C given instead of tryptophan -2-^{14}C

[b] Drug injection 1 hour before radioisotope.

caused a further reduction in tryptophan oxidation. The oxidation of the side-chain-labeled tryptophan was inhibited more than that of the pyrrole-ring-labeled tryptophan by this α-blocker.

It is evident from Table XXIII that there is a greater tendency for the inhibition, mediated by the β-blockers, to diminish within 6 hours. than that of the other group. Pre-treatment at 1 hour with the β-blockers did not lessen the response.

To summarize, a group of α- and β-adrenergic blocking agents reduced the oxidation of tryptophan <u>in vivo</u>. Of the five α-blockers studied only dibenzyline and yohimbine had large and consistent effects, if given at the same time as the tryptophan-^{14}C or 1 hour before. Once again this method of assessing the effects of drugs on tryptophan metabolism allows us to detect the action of an important group of drugs on a particular area of intermediary metabolism. At the same time, the limitations of the method become apparent and other types of experiments are now necessary to evaluate the specificity of these drug effects.

Formate-^{14}C and alanine-^{14}C were injected into rats with a few representative blocking agents (Table XXIV). Only dibenyline lowered the rate of oxidation of formate (10-12%); pronethalol accelerated the conversion of formate to CO_2, while DCI was without effect. The oxidation of alanine was markedly reduced by dibenzyline (66-71% lower), but was

Table XXIV. The effect of adrenergic blocking drugs on Na formate and L-alanine oxidation.

Adrenalectomized rats were injected intraperitoneally with the drug followed by Na formate-^{14}C (3 mc/mM) or L-alanine-u-^{14}C (2 mc/mM). Results are expressed as % of controls:

$$\left(\frac{\%\ ^{14}C \text{ recovered as } {}^{14}CO_2\text{-expt'l}}{\%\ ^{14}C \text{ recovered as } {}^{14}CO_2\text{-control}} \times 100\right)$$

FORMATE-^{14}C

DRUG	DOSE mg/kg	% OF CONTROL AT:		
		2 hrs.	4 hrs.	6 hrs.
DIBENZYLINE	10	88	89	90
PRONETHALOL	30	163	166	164
DCI	30	99	98	97

L-ALANINE-u-^{14}C

DRUG	DOSE mg/kg	% OF CONTROL AT:		
		2 hrs.	4 hrs.	6 hrs.
DIBENZYLINE	10	29	34	33
PHENTOLAMINE	20	91	99	99
PRONETHALOL	30	87	99	96
DCI	30	90	100	100

unaltered by the other blocking agents.

Although phentolamine inhibits the oxidation of tryptophan-3'-^{14}C to a greater degree than tryptophan-2-^{14}C (Table XXII), this cannot be explained on the basis of its effect on alanine metabolism. These results indicate that the adrenergic blocking drugs that reduce the formation of $^{14}CO_2$ from tryptophan-^{14}C do so by a mechanism more specific than that of inhibiting the oxidation of alanine or formate (intermediates of tryptophan metabolism) or altering respiration, with the exception of dibenzyline. The degree to which formate oxidation is inhibited by dibenzyline (10-12%) does not fully account for its effects on tryptophan-2-^{14}C degradation (48-67% inhibition). This issue will be referred to again in the discussion.

Another possibility considered was that the drugs exerted these effects by a blockade of an adrenergic mechanism which consequently altered tryptophan transport or the integrity of the liver cell (e.g. blood flow, Na^+ levels). Therefore, the influence of adrenaline on the inhibition exerted by the blocking agents was studied. The results indicate (Table XXV) that there is no consistent amelioration of the inhibition of tryptophan degradation following administration of adrenaline. In one experiment the inhibition produced by phentolamine was reduced slightly; in another it was increased. Adrenaline relieved partially the effects of dibenzyline and DCI in some but not all experiments.

It can be concluded that the evidence presented does

Table XXV. Effect of L-adrenaline on the inhibition of DL-tryptophan-2-^{14}C oxidation by adrenergic blocking drugs.

Adrenalectomized rats were injected intraperitoneally with the blocking agent. One hour later they were given L-adrenaline (0.5 mg/kg) by the same route followed by DL-tryptophan-2-^{14}C (1.08 mc/mM). Controls received 0.9% NaCl.

DRUG	DOSE mg/kg	ADREN.	% INHIBITION AT: 2 hrs.	6 hrs.
DIBENZYLINE	10	-	62	54
		+	46	23
ROGITINE	20	-	34	17
		+	72	51
ROGITINE	20	-	71	60
		+	53	53
PRONETHALOL	30	-	56	35
		+	62	39
DCI[a]	30	-	0	0
		+	0	35
DCI	30	-	72	36
		+	29	0

[a] DCI - dichloroisoproterenol

not support an effect of adrenergic mechanisms on the tryptophan degrading system, in adrenalectomized rats. The possibility that the adrenergic blocking drugs may inhibit tryptophan pyrrolase specifically was explored: tryptophan pyrrolase activity was measured in vitro following parenteral administration of the drugs, and tryptophan pyrrolase from livers of untreated rats was assayed in the presence of these drugs in vitro (Section 5D). The results with dibenzyline, pronethalol, and DCI, the most potent inhibitors, in vivo, are presented in Table XXVI. DCI reduced tryptophan pyrrolase activity by 29% whereas both dibenzyline and pronethalol were without effect. The activity of the preincubated enzyme was also measured, to determine whether the inactivation of tryptophan pyrrolase which takes place spontaneously was accelerated following administration of these drugs. If endogenous cyclic-AMP does, in fact, protect the enzyme against inactivation, and if the blocking drugs interfere with the synthesis of this nucleotide, then reduced concentrations of the latter in the homogenate would favour a more rapid inactivation of the enzyme. It is evident from Table XXVI that: 1) the drugs did not alter the stability of the enzyme; 2) Measurement of endogenous holoenzyme furnished an estimate of the degree to which the enzyme was saturated with the endogenous cofactor; none of the blocking agents affected the binding of the enzyme with its cofactor; 3) It is interesting to note that DCI lowered

Table XXVI. The effect of administration of dibenzyline, pronethalol and dichloroisoproterenol on tryptophan pyrrolase activity as measured in vitro.

Rats were injected intraperitoneally with dibenzyline.HCl (10 mg/kg), pronethalol.HCl (30 mg/kg) and dichloroisoproterenol.HCl (20-35 mg/kg). Controls received 0.9% NaCl. Two hours later the animals were killed, the liver extracts of two similarly-treated animals were pooled, and assayed for tryptophan pyrrolase.

The homogenates were assayed: (1) immediately, in the presence of hematin (ASSAY); (2) after the preparation was incubated for 30' at 37°C in the absence of substrate (30' INACTIVATION); (3) immediately, without hematin (ENDOGENOUS HOLOENZYME).

TREATMENT	μmoles kyn/gm/hr		
	+ HEMATIN	30' INACT.	-HEMATIN
CONTROL[a]	3.1 ± 0.3	1.1 ± 0.2	1.6 ± 0.3
DIBENZYLINE	3.5, 2.8	1.5, 0.9	2.3, 1.8
PRONETHALOL	3.1	1.1	1.6
DCI	2.1, 2.2	0.7, 1.1	1.2, 1.2

[a] Five sets, 2 livers/set, mean ± S.D.

the activity of the fraction constituting endogenous apoenzyme. This may reflect a more rapid inactivation of the uncombined enzyme *in situ*, a finding that was substantiated by further studies *in vitro* (Section 5d).

By injecting drugs and assaying enzyme activity *in vitro* it is still difficult to conclude whether the measurements are a true reflection of the activity of the enzyme *in situ*. The drugs may assume a spatial arrangement that favours interference with the action of the enzyme and this orientation may be destroyed by homogenizing the tissue or, in the process of diluting the homogenate, the drug concentration may be lowered beyond a point of effectiveness.

Throughout these studies, the rate of oxidation of tryptophan-2-^{14}C was used as the criterion for deciding whether or not the action of the drug was on the tryptophan degrading pathway. As emphasized earlier, the radioactive carbon of this isomer could only be released from the indole nucleus by the action of tryptophan pyrrolase and formamidase.

As most of the drugs studied inhibited this form of radioactive tryptophan, it was assumed that, if the reduced oxidation was due to an inhibition of an enzyme in the degradative pathway, tryptophan pyrrolase, or formamidase was the target enzyme. It therefore seemed necessary to determine the direct effect of the drugs on tryptophan pyrrolase.

In the next chapter these experiments will be described and an attempt will be made to correlate the *in vivo* and *in vitro* results.

5. Tryptophan Pyrrolase Activity In Vitro

Livers from adrenalectomized animals were used as the source of tryptophan pyrrolase. The activity was measured as described under "Methods". Fig. 19 illustrates that after a lag period of 20 minutes, the reaction is linear for as long as 100 minutes. In subsequent work, readings at 60 minutes were used to estimate the enzyme activity. Approximately 50% of added tryptophan was converted to kynurenine in this period of time. The kynurenine in the homogenate declined in the absence of tryptophan signifying an active kynureninase (or transaminase). Therefore at 60 minutes, the contribution of endogenous kynurenine to the absorbance was insignificant. If high concentrations of kynurenine (3-6 µmoles) were added to the incubation medium at the onset of the experiment, 85% or more was recovered at 1 hour. This would indicate that the preparation did not metabolize excess kynurenine efficiently; therefore the estimation of enzyme activity by product formation was not hampered by significant destruction of the product. These curves were very similar to those obtained by Feigelson and Greengard (118). Although some reports have stated that enzyme prepared in this way has no cofactor requirements (118, 160) the activity of this preparation was more than doubled by addition of hematin. The following values (mean ± S.D.) illustrate this point (no. of

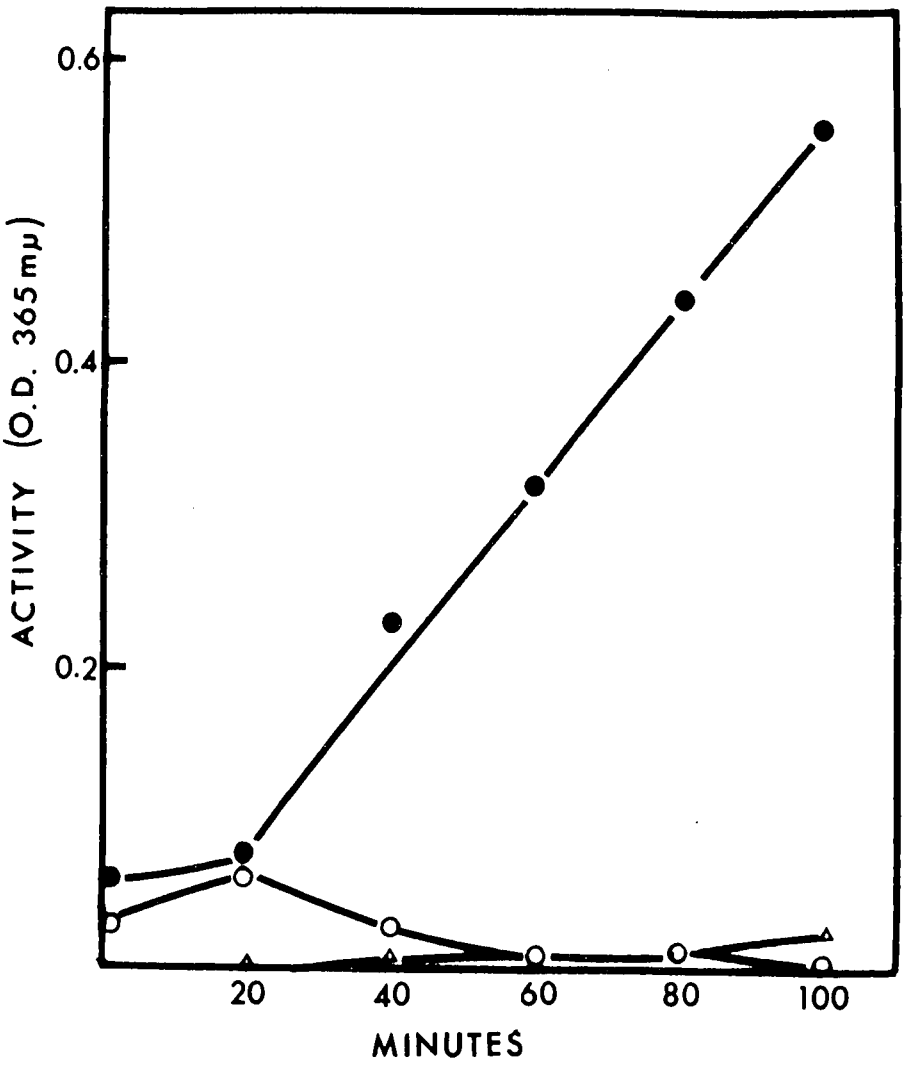


Fig. 19. The time course of the tryptophan pyrrolase reaction.

Tryptophan pyrrolase was assayed in liver of adrenalectomized rats. Liver homogenate (final conc.: 4%) was added to the reaction mixture (Na phosphate buffer, 0.2M, pH 7.0; hematin 7×10^{-6}M; water). The reaction was started by the addition of L-tryptophan (1.3×10^{-3}M) and stopped with metaphosphoric acid (15%). Optical density (365 mμ) was measured in the neutralized filtrates at 20 min. intervals in homogenates with tryptophan added (● - ●) or omitted (○ - ○). A reagent blank was read against water (△ - △).

experiments in parentheses):

	μmoles kyn/g /hr
Hematin added	3.2 ± 0.4 (13)
No hematin added	1.6 ± 0.4 (13)

a. Enzyme Inactivation

The relationship between enzyme concentration and enzyme activity is illustrated in Fig. 20. Enzyme activity was proportional to enzyme concentration in the range of 1.2-6.3% (final concentration of enzyme). If the enzyme was preincubated for 30 minutes without substrate, the activity-concentration relationship was linear over a wider range of values (1.5% - 8.7%). A second experiment with a preparation of lower activity (maximum absorbance: 0.5) yielded identical results. This showed that the non-linearity of the concentration-activity curve in the range of high enzyme concentration, was not due to inaccuracy of the spectrophotometric readings. The rate of spontaneous inactivation of the enzyme was routinely measured in studies on inhibitors to determine whether drugs interact with the enzyme more rapidly in the absence of added substrate, or cause a more rapid decline of enzyme activity under these conditions.

After 30 minutes of preincubation, the activity of tryptophan pyrrolase declined to about one-third of its original value and did not change thereafter(Fig. 21).

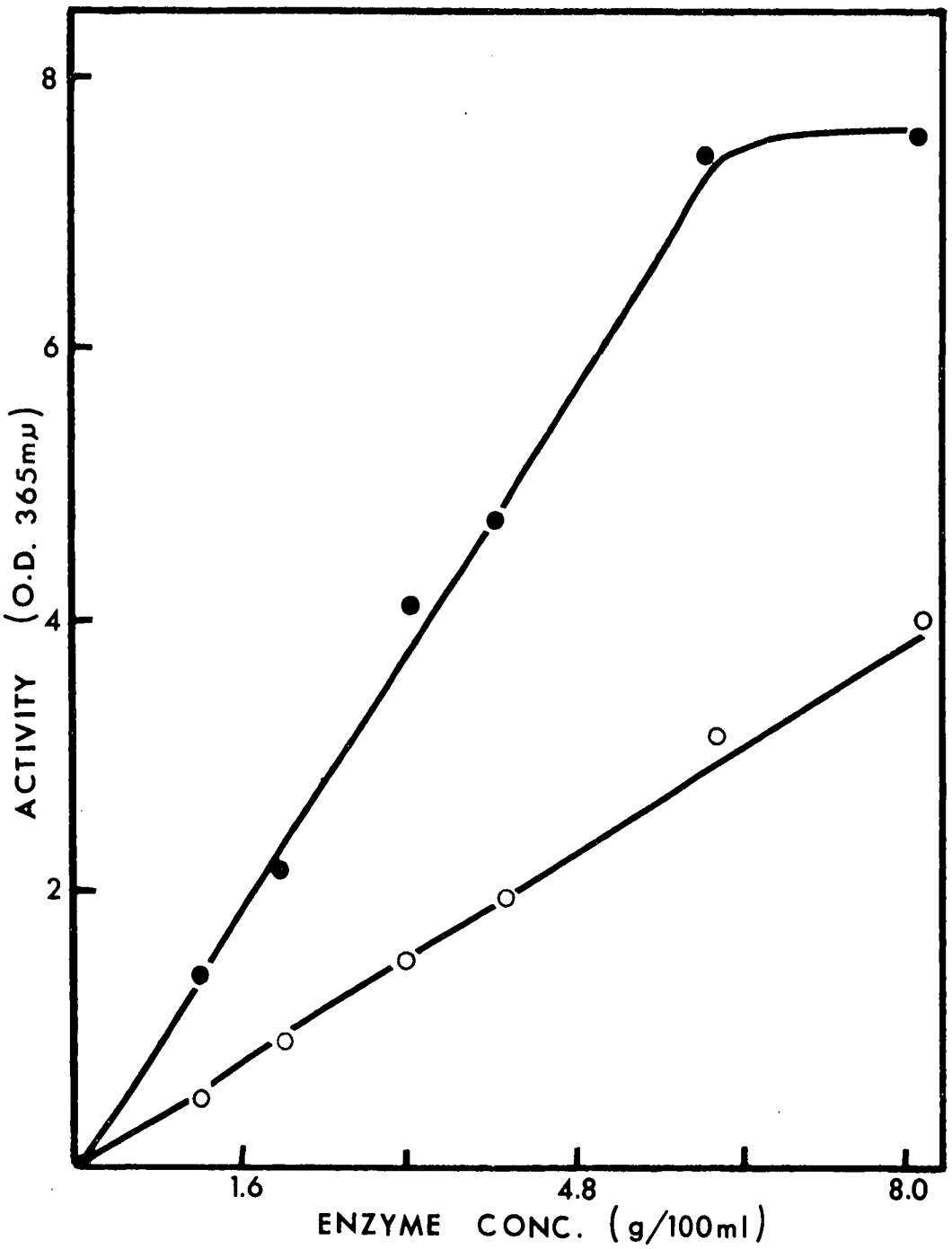


Fig. 20. The relationship between enzyme concentration and enzyme activity.

Increasing amounts of liver homogenate were added to the reaction mixture, described in Fig. 19. The homogenate was assayed immediately (●-●) or after 30' of preincubation at 37°C without substrate (○-○).

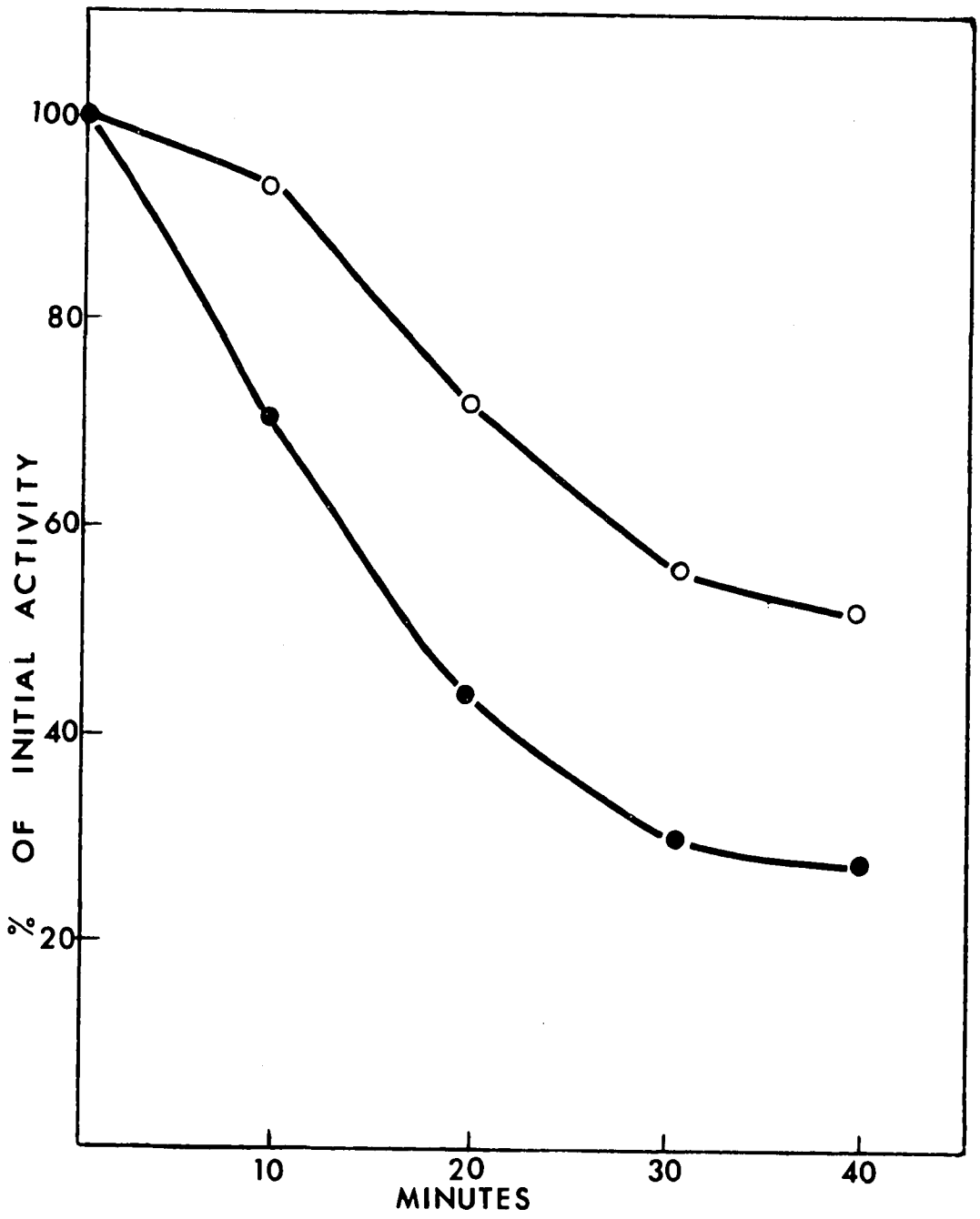


Fig. 21. The time course of inactivation of tryptophan pyrrolase.

Enzyme preparation was preincubated at 37°C without tryptophan (● - ●), without tryptophan but in the presence of α-methyltryptophan, 5×10^{-4}M (O - O). At 10 minute intervals, tryptophan was added to individual reaction vessels which were then incubated for a further 60 min. Details of the assay are given with Fig. 19.

α-Methyl-tryptophan, known to protect the enzyme against spontaneous inactivation (130, 139) reversed the inactivation, partially.

The inactivation occurs only under aerobic conditions (130). Knox and Piras have claimed that the lowered activity following preincubation was due solely to the reoxidation of the reduced (active) form of the cofactor (129). It was important to establish whether hematin is the critical factor in the inactivation process; this would provide further insight into the mechanism of inactivation and would aid in interpreting the results obtained with inhibitors.

Total enzyme (Table XXVII) refers to the endogenous holoenzyme (enzyme already combined with endogenous cofactor) plus the endogenous apoenzyme (enzyme that combines with added hematin). Of the total activity, 69% was eliminated by preincubation. The contribution of endogenous holoenzyme to the total activity was 35% ($\frac{1.5}{4.3}$ x 100 µmoles kyn/gm/hr). The degree of inactivation of this fraction was 80%. The contribution of endogenous apoenzyme is 65%, by subtraction, and this fraction was inactivated 61%. Whereas the endogenous holoenzyme represents 35% of the total activity in the fresh preparation, in the inactivated preparation it represents only 21% of the total activity. Therefore the enzyme that is combined with the endogenous cofactor is inactivated more rapidly than the enzyme-hematin complex. These results would

Table XXVII. The influence of hematin on the inactivation process.

The enzyme preparation was assayed either immediately or after a 30 min. preincubation period at 37°C in the absence of substrate (A). The legend of Fig. 19 describes the reaction medium. The experiment was repeated in the presence of α-methyltryptophan (5×10^{-4}M)(B). Please see text for explanation.

(A)

FORM OF ENZYME	HEMATIN	μmoles kyn/gm/hr		% INACTIVATION
		STANDARD ASSAY	30' PREINCUBATION	
TOTAL ENZYME	+	4.3	1.4	69%
ENDO. HOLOENZ.	-	1.5 35%[c]	0.3 21%[c]	80%
ENDO. APOENZ.[a]	+	2.8 65%[c]	1.1 79%[c]	61%
"TOTAL ENZYME"[b]	(+)	-	0.8	
ENDO. APOENZ.[a]	-	-	0.5	64%

(B)

FORM OF ENZYME	HEMATIN	μmoles kyn/gm/hr		% INACTIVATION
		STANDARD ASSAY	30' PREINCUBATION	
TOTAL ENZYME	+	4.4	2.5	43%
ENDO.HOLOENZ.	-	1.6 36%[c]	1.3 52%[c]	19%
ENDO. APOENZ.[a]	+	2.8 64%[c]	1.2 48%[c]	58%
"TOTAL ENZ." [b]	(+)		2.7	

[a] by subtraction

[b] hematin added at the end of the 30' preincubation period

[c] % of total enzyme activity

suggest that the endogenous cofactor is not identical with hematin. Recent reports have indicated that methemoglobin is a better external source of cofactor than hematin (121, 122).

After preincubating the enzyme system without hematin or substrate, hematin was added and the activity measured. The total activity ("total enzyme", Table XXVII, A) was 0.8 units. The contribution of endogenous holoenzyme to this value was 0.3 units, and, by subtraction, the endogenous apoenzyme provided 0.5 units of activity. This implies that the endogenous apoenzyme - in the absence of hematin - is also inactivated. If it were not, then addition of hematin to the mixture would restore the activity of endogenous apoenzyme fraction to its full value, i.e. 2.8 units of activity. And one would expect the value for "total enzyme" to be 0.3 + 2.8 = 3.1 units, instead of 0.8 units. This provides evidence that the reduction of tryptophan pyrrolase activity, in the absence of sbustrate, is not solely due to hematin oxidation. The proof is that the endogenous apoenzyme is inactivated without hematin present but not as much as holoenzyme; addition of hematin to the system does not restore full activity.

In order to clarify this further, α-methyl tryptophan was used to protect the enzyme. Knox and Piras have stated that the presence of substrate or α-methyl tryptophan pre-

vents the reoxidation of the cofactor and this constitutes the protective mechanism against enzyme inactivation (129). The inactivation of the total enzyme was now only 43% with ∝-methyltryptophan present, as against 67% without it.

The total activity is a composite of the separate activities of endogenous holo- and apoenzyme. ∝-Methyltryptophan almost reversed the inactivation of the endogenous holoenzyme (19% inactivation) but had very little effect on endogenous apoenzyme; in the presence of the analogue the inactivation was 58%, in its absence, 61%. This provides further evidence that the endogenous enzyme-cofactor complex has different characteristics than the endogenous enzyme combined with added hematin. ∝-Methyltryptophan and the enzyme were preincubated without hematin or tryptophan for 30 minutes. The results of this assay (Table XXVII, B, "total enzyme") indicate that hematin is not necessary for the protective mechanism. The activity of the enzyme protected in the presence of hematin was 2.5 units, and in its absence, 2.7 units. This implies that ∝-methyltryptophan does not prevent inactivation by protecting the cofactor against oxidation.

b. Alteration of Tryptophan Pyrrolase Activity In Vitro by Tryptophan Analogues.

In previous sections (4 a, b, c), several aromatic and, in particular, indole derivatives were shown to reduce

the rate of oxidation of tryptophan *in vivo*. This section describes the effects of some of these compounds on tryptophan pyrrolase *in vitro*.

Of the tryptophan derivatives studied, ∝-hydrazinotryptophan and tryptophol were the most potent inhibitors of the enzyme (Table XXVIII). Hypaphorine was a weak inhibitor of tryptophan pyrrolase at a concentration of 10^{-3}M. All three ring-hydroxylated derivatives of tryptophan lowered tryptophan pyrrolase activity *in vitro* to about the same extent whereas *in vivo*, ∝-hydrazino-5-hydroxytryptophan antagonized tryptophan metabolism to a greater degree than ∝-methyl-5-hydroxytryptophan or 4-hydroxytryptophan. Several tryptamine derivatives, which diminish the rate of tryptophan oxidation *in vivo* acted similarly *in vitro*: N-acetyltryptamine decreased the activity of the enzyme by 79% while tryptamine, ineffective *in vivo*, halved it. The tertiary amine, N,N-diethyltryptamine, the ∝-substituted tryptamines (∝-methyl-, ∝-ethyl-) and indole-3-acetic acid were weak inhibitors of the enzyme.

It can be concluded that all derivatives which decrease the oxidation of tryptophan *in vivo* inhibit tryptophan pyrrolase activity *in vitro*. Some compounds that were weakly inhibitory *in vivo* (e.g. tryptophol, ∝-methyl-5-hydroxytryptophan) were very potent inhibitors of tryptophan pyrrolase. Slow transport of these compounds into the liver may account for this discrepancy. A complete comparison of the two sets of results

Table XXVIII. The effect of tryptophan derivatives on tryptophan pyrrolase activity in vitro.

The assay medium was the same as described in Fig. 19. If the enzyme was preincubated at 37°C for 30 minutes, the inhibitor - but not the substrate - was present in the incubator medium.

ANALOGUE	% INHIBITION		
	NO PREINC.		30' PREINC.[a]
	5×10^{-4}M	10^{-3}M	10^{-3}M
α- METHYLTRYPTOPHAN	0%	0	0
α-HYDRAZINOTRYPTOPHAN	51%	75%	81%
HYPAPHORINE	7%	18%	-
TRYPTOPHOL	86%	92%	95%
α-METHYL-5-HYDROXYTRY.	15%	36%	-
α-HYDRAZINO-5-HYDROXYTRY.	27%	50%	58%
4-HYDROXYTRYPTOPHAN	-	51%	-
TRYPTAMINE		45%	77%
N-ACETYLTRYPTAMINE		79%	23%
N,N-DIETHYLTRYPTAMINE		24%	23%
α-METHYLTRYPTAMINE		21%	31%
α -ETHYLTRYPTAMINE		24%	46%
INDOLE-3-ACETIC ACID		24%	0%
HYDRAZINE		27%	65%

[a] Calculated on the basis of the activity of the preincubated enzyme.

will be given in the discussion.

It is possible to draw some conclusions concerning the structural characteristics of these inhibitors: 1) hydroxylation of the benzene ring alters the action of tryptophan and its derivatives: Tryptophan, if hydroxylated on the 4-position of the ring, interferes with the action of tryptophan pyrrolase. 5-Hydroxylation of α-methyltryptophan renders this compound an inhibitor of the enzyme at 10^{-3}M whereas α-hydrazino-tryptophan, if hydroxylated on the 5- position, is a weaker inhibitor than the parent compound. 2) The amino group on the side chain is not necessary for an inhibitory effect; tryptophol and indole-3-acetic acid both lowered the activity of the enzyme, the alcohol being 3 times as active as the acid.

If inhibition of the enzyme is greater following preincubation with an inhibitor, this would suggest that the time of formation of the EI complex is appreciable and that preincubation, therefore, favours the attainment of equilibrium of the complex. If the inhibition is less, then the compound either protects the enzyme against spontaneous inactivation, or it may be destroyed during the preincubation.

Several compounds were preincubated with the enzyme for 30 minutes in the absence of tryptophan. α-Hydrazinotryptophan, its 5-hydroxy derivative, tryptamine, α-methyltryptamine, and α-ethyltryptamine, each containing a primary amino group, caused a further reduction in the activity of the

enzyme on preincubation (Table XXVIII). The degree of inhibition remained unchanged if the enzyme was preincubated with tryptophol or N,N-diethyltryptamine. The indole amide, N-acetyltryptamine, and indole-3-acetic acid inhibited the enzyme to a lesser extent (or not at all) if preincubated with it. These results indicate that the inhibitors react with the enzyme in more than one way. Some inhibitors react more extensively with tryptophan pyrrolase if allowed to incubate with it for a period of time, hence the inhibition is increased. These compounds may have other actions on the enzyme, e.g. protection against spontaneous inactivation which may not be manifested if the inhibitor is not removed from the enzyme by dilution. Other inhibitors require only a few minutes to exert their complete effect (tryptophol, N,N-diethyltryptamine), suggesting that they either come to equilibrium with the active site of the enzyme rapidly, or produce a rapid irreversible inactivation of the enzyme. A third group of inhibitors is less effective if preincubated with the enzyme. This group includes indole-3-acetic acid and N-acetyltryptamine. This may signify that they are: 1) destroyed during the preincubation period or 2) they protect the enzyme against inactivation or 3) convert latent (122) tryptophan pyrrolase to the active form. Hydrazine lowered tryptophan pyrrolase activity by 27%; if preincubated in the absence of substrate the activity was reduced even further, to 65%. It can be concluded that α-hydrazinotryptophan inhibits the enzyme

by a mechanism which is structure-specific, not only by a process brought about by the highly reactive hydrazine group. This indole was about three times as active as hydrazine alone. Hydrazine may lower the activity of the enzyme by reducing the Fe^{+++} of hematin at the onset of the reaction, accepting Feigelson's hypothesis (124). As tryptophan aids in the binding of hematin to the enzyme (123) preincubation in the absence of tryptophan may increase the susceptibility of hematin to attack by hydrazine.

Further studies with α-hydrazinotryptophan and tryptophol were done in order to characterize more completely the inhibition brought about by these derivatives. Both compounds inhibit the enzyme non-competitively (Fig. 22, A). Although tryptophol is less effective than the hydrazino analogue at low concentrations, in the range of 5×10^{-4}M - 10^{-3}M it is a more potent inhibitor (Fig. 22, B). α-Hydrazinotryptophan is more effective than its 5-hydroxy- derivative throughout the entire range of concentrations studied.

α-Hydrazinotryptophan inhibited the endogenous holoenzyme to a lesser extent than the enzyme combined with hematin. The activity of the endogenous holoenzyme was reduced 54%, while the activity of a hematin-supplemented preparation was lowered by 75%. By subtraction, that fraction of the activity representing endogenous apoenzyme would be inhibited 83%. This may indicate that the hydrazino compound prevents the binding of hematin to apoenzyme by

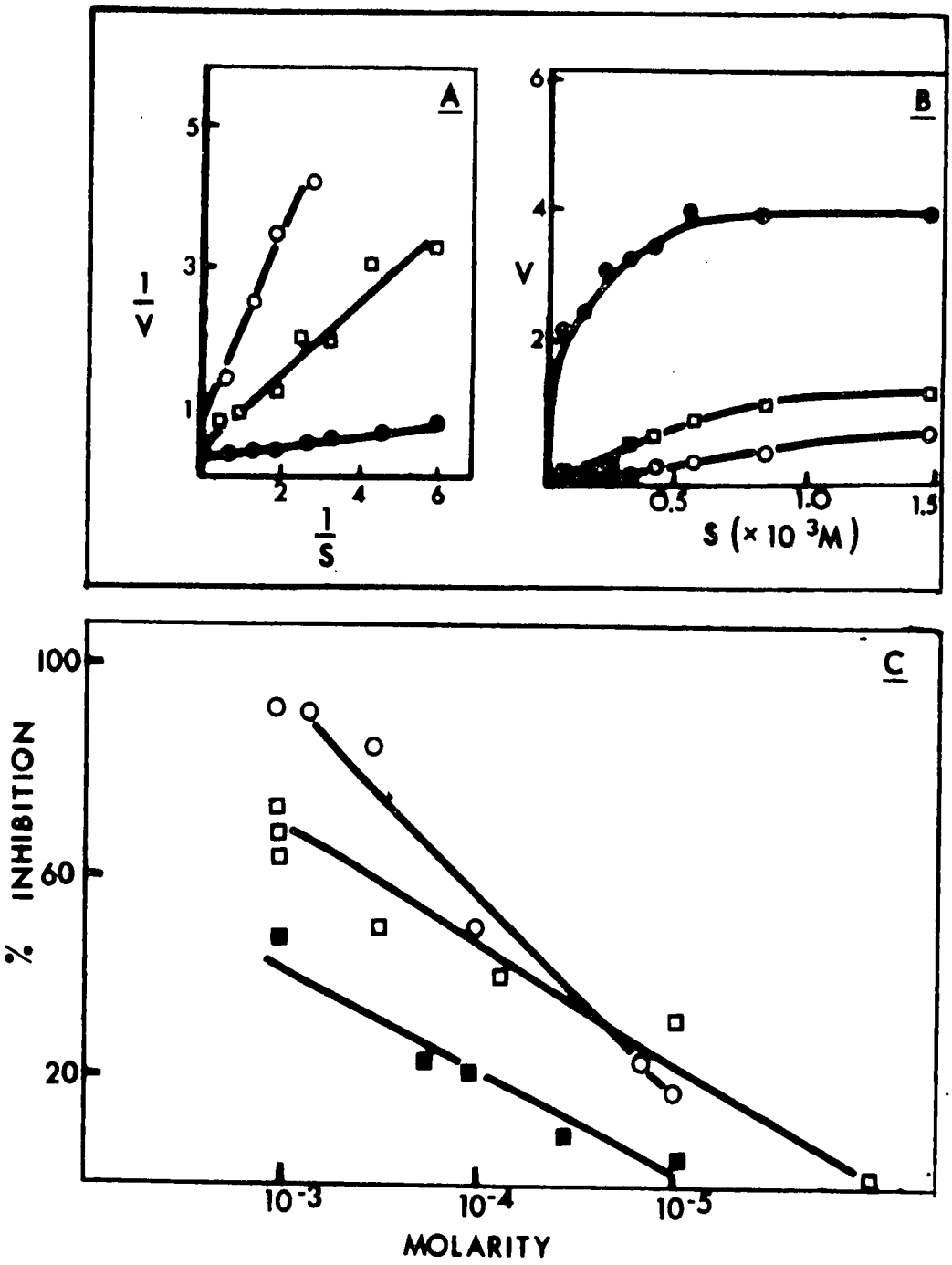


Fig. 22. A,B. The effect of α-hydrazinotryptophan and tryptophol on the rate response of tryptophan pyrrolase to substrate concentration.

Tryptophan pyrrolase was assayed alone (● - ●), in the presence of α-hydrazinotryptophan, 5×10^{-4}M (□ - □) and tryptophol, 5×10^{-4}M (○ - ○). A Lineweaver-Burk plot of the data is given in A.

C. The effect of increasing dose of α-hydrazinotryptophan, α-hydrazino-5-hydroxytryptophan and tryptophol on tryptophan pyrrolase activity. α-Hydrazinotryptophan (□ - □), α-hydrazino-5-hydroxytryptophan (■ - ■), tryptophol (○ - ○).

reacting with it, or with the apoenzyme, or that the apoenzyme-hematin complex is not identical with the endogenous holoenzyme. If the hematin concentration was doubled; the degree of inhibition remained the same.

The possibility that α-hydrazinotryptophan was affecting formamidase activity was examined. This could be done by the principle that inhibition of formamidase would lead to a build-up of formylkynurenine, which absorbs at 321 mμ, resulting in a reduction in the ratio of absorbances at 365: 321 mμ. The ratio of the absorbances at 365 mμ, 321 mμ was calculated for the control:2.5. α-Hydrazinotryptophan did not alter this ratio (2.5) whereas formanilide, a known inhibitor of formamidase reduced it - 2.0, signifying accumulation of formylkynurenine.

c. Alteration of Tryptophan Pyrrolase Activity In Vitro by Indole Alkaloids

Previous studies (Section 4b) showed that some indole alkaloids antagonized tryptophan metabolism in vivo. An attempt to correlate these results with the effect of indole alkaloids on tryptophan pyrrolase in vitro is described here. A complete comparison of in vivo, in vitro results will be given in the discussion.

The concentration of the ergot derivatives used was in the range of 3×10^{-5} - 10^{-4}M. Higher concentrations were not

employed because of limited amounts of the drugs. At these concentrations the ergot alkaloids were without effect on tryptophan pyrrolase (Table XXIX). Both noribogaine and yohimbine interfered with the conversion of tryptophan to kynurenine in vitro (and inhibited tryptophan oxidation in vivo). Corynanthine, a geometric isomer of yohimbine, was a less effective inhibitor (13%) than yohimbine (53%). Igogaine and ibogamine did not diminish the rate of oxidation of tryptophan in vitro (or in vivo). Harmaline interfered with the assay procedure and is therefore not considered. The results indicate that there is a good correlation between those indole alkaloids which retard the conversion of tryptophan-$^{14}C \longrightarrow {}^{14}CO_2$ and inhibit tryptophan pyrrolase activity.

Once again a difference was found in the behaviour of the indole derivatives when preincubated with the enzyme in the absence of substrate. Yohimbine was a more effective inhibitor under these conditions as opposed to noribogaine. Yohimbine was selected for further study as it was the most powerful inhibitor of the alkaloids. The response of tryptophan pyrrolase to increasing substrate concentration in the presence of this alkaloid was determined (Fig. 23). A Lineweaver-Burk plot of the results is characteristic of non-competitive inhibition. While preparing the incubation medium it was noted that a solution of hematin and yohimbine turned dark. The characteristic spectrum of hematin was shifted toward higher wavelengths in the presence of yohimbine but no new

Table XXIX. The effect of indole alkaloids on tryptophan pyrrolase activity *in vitro*.

See Table XXVIII for description.

ALKALOID	FINAL CONC.	% INHIBITION	
		NO PREINC.	30 min. PREINC.
ERGOTAMINE	10^{-4}M	0	-
DIHYDROERGOTAMINE	10^{-4}M	0	-
L.S.D.[a]	3×10^{-5}M	16%,0	-
IBOGAINE	10^{-3}M	0	0
IBOGAMINE	10^{-3}M	0	-
VOBASINOL	10^{-3}M	0	-
NORIBOGAINE	10^{-3}M	39%	25%
"	3×10^{-4}M	21%	0
TABERNANTHINE	10^{-3}M	0	-
YOHIMBINE	10^{-3}M	53%	69%
"	10^{-3}M	52%	70%
CORYNANTHINE	10^{-3}M	13%	-

[a] Lysergic acid diethylamide

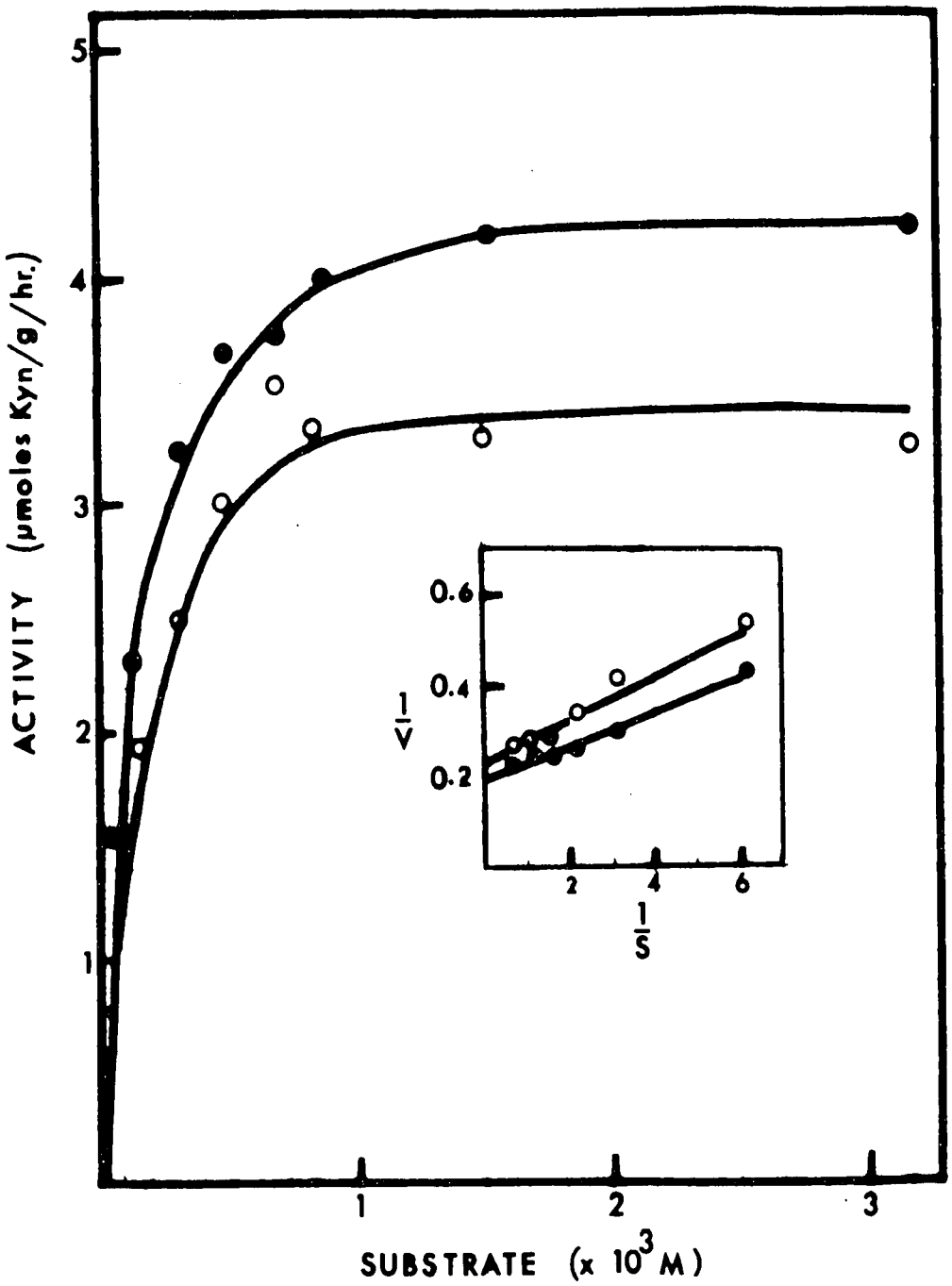


Fig. 23. The rate response of tryptophan pyrrolase to substrate in the presence of yohimbine.

The activity of tryptophan pyrrolase in the absence (● - ●) or presence of yohimbine, 10^{-3}M (○ - ○) is expressed as μmoles kyn/gm/hr. In inset is a Lineweaver-Burk plot of the results.

peaks were observed. It was of interest to determine whether yohimbine lowered the activity of tryptophan pyrrolase by altering hematin (Table XXX). In the standard assay procedure yohimbine lowered the activity of the enzyme 49%. In the absence of hematin yohimbine had no effect on the enzyme (endogenous holoenzyme). In the presence of a preparation highly saturated with endogenous cofactor (i.e. from tryptophan-treated rats) yohimbine was far less effective (Table XXX). This finding suggested that yohimbine interfered with the binding of hematin to the enzyme. When the enzyme was incubated with hematin and tryptophan for 5 minutes and then yohimbine was added, the latter had no effect on the final activity. This would suggest that once the enzyme-cofactor-substrate complex was formed, yohimbine could not alter the enzyme-bound hematin. When the enzyme was incubated with hematin, and then yohimbine was added, followed by the substrate, once again yohimbine was ineffective implying that the hematin bound to the enzyme -- in the absence of substrate-- also was not susceptible to attack by yohimbine. The previous Table (XXIX) illustrated that yohimbine decreased the activity of the enzyme further if preincubated with it. However, if yohimbine was added 4 minutes after the enzyme, it did not lower the activity at all. These results indicate that the further reduction of activity which occurs if the enzyme is preincubated with yohimbine for 30 minutes is also related to its effect on hematin. Corynanthine, a geometric isomer

Table XXX. The effect of yohimbine on the activity of tryptophan pyrrolase.

Tryptophan pyrrolase was assayed as described in Fig. 19. The order of addition of constituents was: hematin, yohimbine (10^{-3}M), enzyme, tryptophan, unless otherwise stated.

FIRST ADDITION	SECOND ADDITION	THIRD ADDITION	FOURTH ADDITION	μmoles kyn / gm/hr	% INHIBITION
HEMATIN	-	ENZYME	TRYPTOPHAN	3.3	-
"	YOHIMBINE	"	"	1.7	49%
HEMATIN[a]	-	ENZYME	TRYPTOPHAN	4.9	-
"	YOHIMBINE	"	"	4.3	12%
-	-	ENZYME	TRYPTOPHAN	1.3	-
-	YOHIMBINE	"	"	1.4	0
HEMATIN	ENZYME	TRYPTOPHAN	-	3.2	-
"	"	"	YOHIMBINE	3.2	0
HEMATIN	ENZYME	-	TRYPTOPHAN	3.6	-
"	"	YOHIMBINE	"	3.5	0
HEMATIN[b]	ENZYME	-	TRYPTOPHAN	1.1	-
"	"	YOHIMBINE	"	1.1	0

[a] Enzyme preparation from adrenalectomized rats administered tryptophan (1 mmole/kg) 2 hours before death

[b] Enzyme preincubated 30 minutes with hematin and yohimbine

of yohimbine, inhibited the endogenous apoenzyme-hematin fraction and endogenous holoenzyme equally. This would indicate that the effect of yohimbine is related to its specific conformation.

d. Alteration of Tryptophan Pyrrolase Activity In Vitro by α- and β-Adrenergic Blocking Drugs

In Section 4d, both α- and β-adrenergic blocking drugs were shown to inhibit the rate of metabolism of tryptophan. It was suggested that this effect may be a direct or indirect one -- the former being due to inhibition of specific enzymes along the degradation pathway of tryptophan, the latter being related to an adrenergic blocking mechanism. It was considered that if enzyme inhibition were solely responsible for the observed results, then tryptophan pyrrolase may be the enzyme or one of the enzymes involved.

The effects of three α- blockers structurally unrelated to tryptophan on tryptophan pyrrolase activity were studied. All three, dibenzyline, dibenamine and phentolamine, inhibited the enzyme in vitro (Table XXXI).

Dibenzyline and dibenamine, two alkylating agents that are related chemically, both reduced the activity of the enzyme to the same extent. These compounds were non-competitive antagonists of the enzyme (Fig. 24). An attempt was made to outline further the specificity of action of these drugs. One

Table XXXI. The effect of adrenergic blocking agents on tryptophan pyrrolase activity.

The enzyme, prepared by pooling four livers of adrenalectomized rats, was assayed in the whole homogenate (H) or the supernatant (S) fraction as described in Fig. 19. Preincubation of the enzyme was carried out at 37°C for 30 minutes. The final concentration of the homogenate was 4-5% unless otherwise indicated. Drug conc.: 10^{-3}M.

α-Adrenergic Blocking Agents

DRUG	ENZYME PREP.	% INHIBITION	
		NO PREINCUBATION	30 min. PREINC.
DIBENZYLINE	H	65%	81%
	S	58%	99%
DIBENAMINE	H	66%	80%
	S	60%	90%
PHENTOLAMINE	H	19%	25%
	S	25%	40%
β-Adrenergic Blocking Agents			
PRONETHALOL	H	0	41%[a] (25-61)[b]
	S	0	0
	H[c] or S[c]	0	0
PROPRANOLOL	H	0	21% (0-38)[b]
	S	0	0
	H[c] or S[c]	0	0
DCI	H	0	33% (0-56)[b]
	S	0	0
	H[c] or S[c]	0	0

[a] mean of three experiments
[b] range of values
[c] 2% homogenate (final conc.)

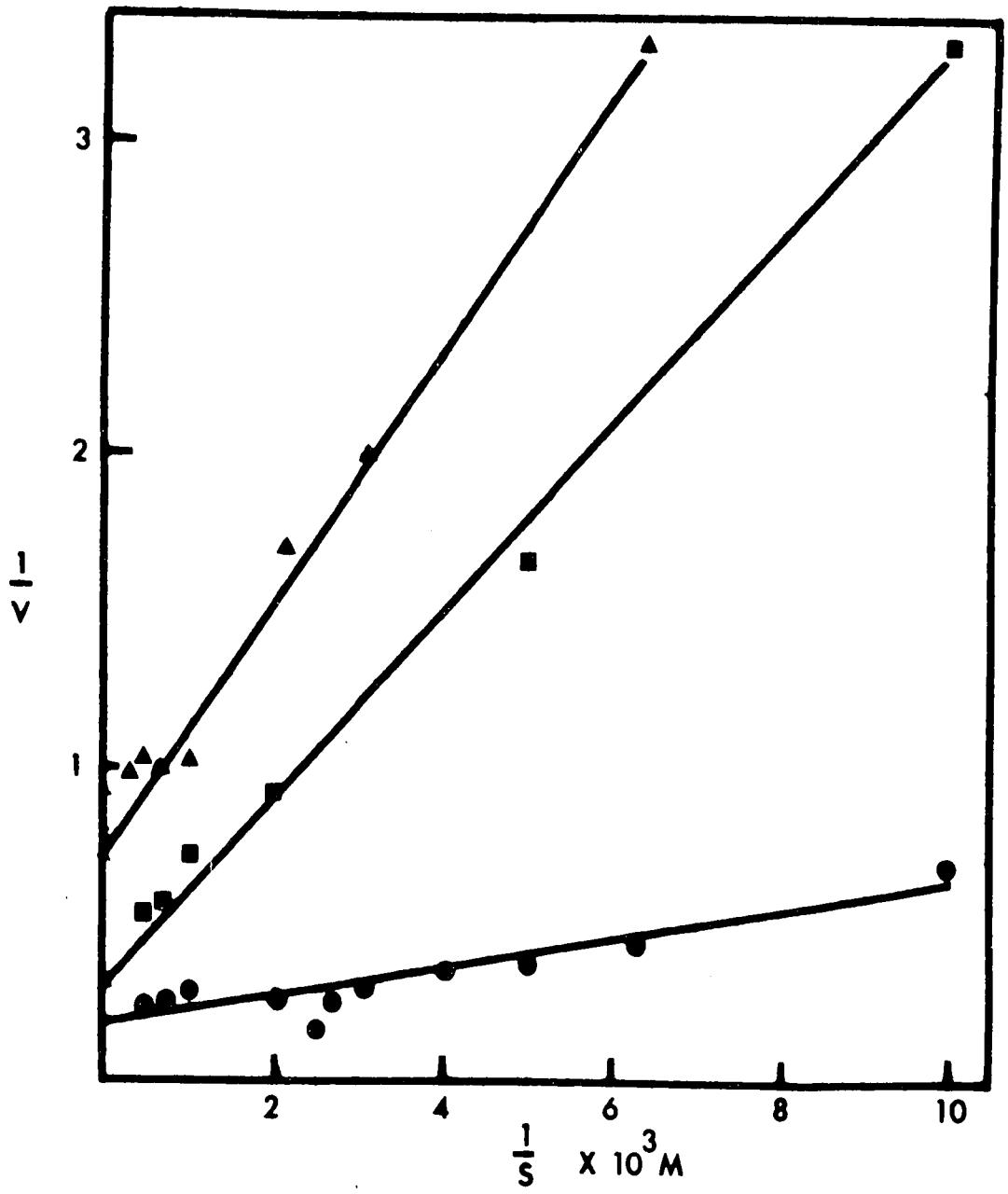


Fig. 24. The rate response of tryptophan pyrrolase to increasing substrate concentration in the presence of dibenzyline and dibenamine.

Tryptophan pyrrolase activity was measured as described in Fig. 13. Final conc. of the homogenate was 4%. Dibenzyline (▲ - ▲) and dibenamine (■ - ■), both at a concentration of 10-3M, are compared to the untreated preparation (● - ●).

of the possibilities considered was that dibenzyline and dibenamine act on formamidase thereby reducing the formation of kynurenine by an effect other than on tryptophan pyrrolase. Formylkynurenine, the substrate of formamidase and precursor of kynurenine, absorbs at 321 mμ. Following incubation of the enzyme and substrate with dibenzyline, the absorbance at 321 mμ did not increase with respect to the absorbance at 365 mμ (kynurenine) indicating that there was no accumulation of formylkynurenine, hence no inhibition of formamidase.

Another possibility considered was that the drugs react chemically with the kynurenine formed, thereby reducing its absorbance. However, if kynurenine was added to the incubation medium at the onset of the reaction it was recovered quantitatively. This shows that the blocking drugs did not cause an apparent reduction of the activity of tryptophan pyrrolase by altering the product of the reaction.

In the previous section it was indicated that yohimbine may exert its effect by preventing the combination of hematin and enzyme:

Yohimbine exerted no influence on the enzyme preparation to which no hematin was added but did inhibit the preparation containing hematin. This possibility was explored with dibenzyline and dibenamine. Both exerted similar inhibitory effects in the presence or absence of hematin. The results suggest that these blocking agents inhibit the enzyme by a

mechanism unrelated to 1) the binding of hematin to the enzyme, or 2) an alteration of the characteristics of hematin.

Additional studies were carried out using different preparations of the enzyme. Both dibenzyline and dibenamine were preincubated with the enzyme -- contained in the whole homogenate -- to see whether they cause a further decrease in the activity of the enzyme under these conditions. As in the case of certain tryptophan analogues (Section 5b) and indole alkaloids (Section 5c) both blocking agents reduced the activity of the enzyme further. However, if preincubated with a supernatant fraction, the loss in activity was greater (90, 99%) than the loss in activity of the whole homogenate (80, 81%). This discrepancy was not evident if the blocking drugs were not preincubated with the enzyme; that is, both dibenzyline and dibenamine inhibited the enzyme contained in the supernatant fraction or whole homogenate to the same extent if added immediately before the onset of the reaction. Assuming that the reaction between the drugs and the enzyme is time-dependent, it follows that the preincubated enzyme would be inhibited more than the enzyme assayed immediately. One can explain the greater loss in activity of the preincubated supernatant fraction with respect to the whole homogenate on the basis of the non-specificity of the alkylating agents. Thus in a whole homogenate more protein is present, which can react with the drugs. Removal of the particulate fraction

results in more of the alkylating agent being available for reaction with tryptophan pyrrolase.

Phentolamine was less effective than the haloalkylamines at equimolar concentrations. The drug was incubated in the absence and presence of hematin and found to inhibit both preparations to the same extent (21%, 19%, resp.). Thus, like dibenzyline and dibenamine, its action is unrelated to phenomena concerned with hematin - enzyme complex formation. It also inhibited the enzyme further if preincubated with it for 30 minutes. Once again, the activity in the supernatant fraction was inhibited to a greater extent than the activity of the whole homogenate if allowed to preincubate with phentolamine.

The results indicate that some α-adrenergic blocking agents inhibit tryptophan oxidation in vitro. The actions of the non-indolic drugs, dibenzyline, dibenamine and phentolamine, on tryptophan pyrrolase are similar in the following respects: (a) the mechanism of inhibition is unrelated to hematin; (b) the drugs react more extensively with the enzyme if preincubated with it.

In sharp contrast to the above findings, the β-adrenergic blocking drugs (Table XXXI) had no effect on the fresh preparation of enzyme.

If preincubated with the enzyme (final concentration 4-5%) in the absence of substrate, pronethalol, propranolol

and dichloroisoproterenol lowered the activity of the enzyme in a majority of experiments. If a more dilute preparation of the enzyme was used (2%) the loss in activity did not occur. Moreover if the supernatant was preincubated with the drugs, instead of the whole homogenate, the reduction in activity was not observed. This may indicate that a slow reversible reaction takes place between the drugs and the enzyme, if the enzyme is not in "catalytic conformation". Reduction of the available enzyme, by dilution, or by inactivation (supernatant), or by occupation with substrate, lessens the degree of this reaction.

It is unlikely that the β -adrenergic blocking drugs retard tryptophan metabolism in vivo by this mechanism.

III. DISCUSSION

A. Catabolism of Tryptophan in Vivo.

Studies on the distribution of L-tryptophan (Fig. 9) support the findings of several investigators (22, 23, 91), that administered tryptophan is metabolized very rapidly. Within 6 hours no excess xanthydrol-reacting material was detectable in serum, liver, kidneys, and brain. The pancreas retained elevated levels of the amino acid, and/or its metabolites, for at least one day.

Using a specific method for measurement of tryptophan, Civen and Knox noted its disappearance from liver and serum within 6 hours (22).

The accumulation of L-tryptophan by the brain substantiates the earlier studies of Guroff and Udenfriend (213). Although brain tryptamine and serotonin increase following administration of tryptophan (53), the decline of the amino acid in the brain is probably a result of protein incorporation and equilibration with blood rather than metabolism along these minor pathways. Because the pancreas does not contain pyrrolase, and is a site of rapid protein synthesis, a large accumulation of tryptophan in the organ is to be expected.

The disappearance of tryptophan from tissues is , in part, a result of its catabolism along the pyrrolase pathway.

In the work reported herein, functioning of this pathway was determined by the rate of conversion of tryptophan-^{14}C to

$^{14}CO_2$ *in vivo*. Tryptophan, labeled with ^{14}C in different positions, was employed. A comparison of the rates of evolution of $^{14}CO_2$ from different radioisomers of tryptophan enables deductions to be made concerning the activity of steps along the degradative route under varying conditions; thus the enzyme steps which can become rate-limiting in the whole animal after a tryptophan load or metabolic inhibitors, or in vitamin-deficiency, may be detectable this way. Moreover, multiple sites of action of inhibitors can be determined.

Among the difficulties encountered with this method are the possible effects of tryptophan-^{12}C or drugs on the transport of tryptophan-^{14}C, drug effects on respiration, reutilization of evolved $^{14}CO_2$, and isotope dilution. In the course of this work, an attempt has been made to detect transport and respiratory effects of drugs, and possible sites of isotope dilution.

L- and D-Tryptophan oxidation

Throughout the studies *in vivo*, DL-tryptophan-^{14}C was used (except in the case of L-tryptophan-$^{14}COOH$). This raises the question of what contribution D-tryptophan makes to the expired $^{14}CO_2$ and by what metabolic route. It is known that D-tryptophan is transported as rapidly as the L- form into the blood following intraperitoneal administration (213). In tissues, the D- form may be metabolized along two routes: inversion to the L- form by oxidative deamination and subsequent reamination,

or direct oxidation to D-kynurenine. The former route is efficient as was borne out by a comparison of the rates of oxidation of DL- and L-tryptophan-^{14}COOH; both contributed equally to respiratory $^{14}CO_2$. Although there is increasing evidence that D-tryptophan is oxidized in a manner analogous to the L-isomer (38, 127) this route would probably not contribute significantly to respiratory $^{14}CO_2$: D-Kynurenine is not a substrate for kynureninase *in vitro* (63) and, *in vivo*, only 2-3% of administered D-kynurenine-^{14}C and D-3-hydroxykynurenine-^{14}C can be accounted for in respiratory gases within 6 hours. On the other hand, 70%-90% of the L- forms of these amino acids are converted to $^{14}CO_2$ (78, 79). It is therefore assumed that the contribution of the D-isomer to respiratory $^{14}CO_2$ is similar to that of L-tryptophan (with physiological doses) and is a result of its inversion to the L-isomer.

A comparison of the rates of catabolism of tryptophan-2-, -3'-, and benzene-^{14}C indicates that, with physiological doses (10 μM/kg), all three forms of radioactive tryptophan are oxidized at similar rates (Table I). As the rates of catabolism of the three radioisomers of tryptophan will be referred to, it is pertinent to outline the pathway leading to the formation of $^{14}CO_2$ from each of these forms of tryptophan.

The only known tryptophan-degrading enzyme in mammalian tissue that can initiate the conversion of tryptophan-2-, -3'-, or -benzene-^{14}C $\longrightarrow$ $^{14}CO_2$ is tryptophan pyrrolase. Following

oxidation of the pyrrole ring by pyrrolase and formamidase, formate-^{14}C is released from tryptophan-2-^{14}C. Formate-^{14}C is metabolized in 3 ways: 1) oxidation $\longrightarrow$ $^{14}CO_2 + H_2O$; 2) excretion in urine as the Na salt; 3) activation and subsequent utilization as a C_1 unit. Malorny *et al.* have calculated the biological half-life of formate as 81.5 min. (214). According to the results of the author 50% of exogenous formate is converted to $^{14}CO_2$ in 2 hours. It cannot be stated with certainty that the half-life of exogenous formate is equivalent to that released from tryptophan. Nevertheless, the liver does contain an active formate-oxidizing system and it is therefore assumed that the rate of oxidation of formate would not become a limiting factor in the measurement of tryptophan-2-^{14}C catabolism *in vivo*.

The rates of oxidation of tryptophan-2- and -3'-^{14}C are equivalent. This could be a consequence of two factors: the rate of oxidation of alanine and formate derived from tryptophan are similar and no rate-limiting steps leading to the formation of anthranilic acid or 3-hydroxyanthranilic acid exist. Exogenous alanine-^{14}C and formate-^{14}C are metabolized at comparable rates, 50% of both appearing in respiratory gases within 2 hours. Once again a parallel metabolism of exogenous and endogenous intermediates cannot be assumed. Nevertheless these observations are in accord with the similar rates of catabolism of T_2 and $T_{3'}$. Administered kynurenine or 3-hydroxykynurenine did not decrease respiratory $^{14}CO_2$ from tryptophan-3'-^{14}C, and this supports the possibility that

kynurenine hydroxylase and kynureninase are not rate-limiting enzymes in the whole animal. Is kynureninase rate-limiting in B_6-deficiency if presented with physiological concentrations of substrate? The buildup of kynurenine, 3-hydroxykynurenine and xanthurenic acid in B_6-deficient rats treated with tryptophan (67) points to the possibility that it can become rate-limiting. Moreover, the activity of the enzyme in liver of B_6-deficient rats is reduced (62, 65, 69). However, there is no direct evidence that the function of this enzyme is less in vitamin B_6-deficiency, with physiological doses of substrate. Thus Korbitz et al. found equivalent amounts of anthranilic acid in urine of controls and B_6-deficient animals (67).

Rats placed on a vitamin B_6-deficient diet were unable to metabolize DL-tryptophan-3'-^{14}C (or tryptophan-benzene-^{14}C) as rapidly as controls (Table VIII): the degree of impairment, 30%-40%, rose to 70% when a load of tryptophan was administered along with the radioisotope. This can be a consequence of impaired transport or a reduction in the activity of at least 3 enzymes. Transport was ruled out as a factor because tryptophan-2-^{14}C oxidation was not affected. (1) Tryptophan transamination: it has already been indicated that D-tryptophan contributes to expired $^{14}CO_2$ after its inversion to the L-form. Although the reamination of indolepyruvic acid is a B_6-dependent step, the rate of evolution of $^{14}CO_2$ from DL-tryptophan-2-^{14}C was not altered during the deficiency. Thus the dimi-

nished oxidation of $T_{3'}$ and T_B was not a consequence of impaired inversion of the D- to the L- forms. (2) Alanine transamination: alanine, removed intact from the side chain is transaminated to pyruvic acid by a B_6-dependent system. B_6-deficient rats did expire less $^{14}CO_2$ from alanine-u-^{14}C than controls, confirming other workers (209). The degree of impairment was 15%, but tryptophan oxidation was lowered by 30-40%. Therefore the reduction in tryptophan-3'-^{14}C catabolism cannot be accounted for solely by impairment of the metabolism of non-aromatic intermediates.

Thus by elimination of the other possibilities it can be concluded that tryptophan catabolism is lowered in B_6-deficiency partly as a consequence of the diminished activity of kynureninase. Gholson et al. found a decreased conversion of tryptophan-7α-$^{14}C \longrightarrow {}^{14}CO_2$ in B_6-deficiency (84) which they interpreted as indicative of impairment of tryptophan catabolism at the level of kynureninase. However, they used very high doses of tryptophan in contrast to the doses used here. Even with physiological doses of tryptophan this method of assessing tryptophan catabolism is sufficiently sensitive to detect a reduction in the activity of kynureninase in vivo. Other B_6-enzymes, if present in the catabolic pathway of tryptophan, may also account for these observations.

The apparent rate of oxidation of the 6 ^{14}C's from the benzene ring of tryptophan was higher than the other forms

of radioactive tryptophan (T_2, T_3), but not significantly so (Table I). This is somewhat surprising because the opportunity for isotope dilution is greater with the labeled intermediates from this form of radioactive tryptophan. An outline of the pathway for the oxidation of the benzene ring may help to clarify this (Fig. 3).

Following oxidation by 3-hydroxyanthranilic acid oxygenase the labeled intermediate (α-amino-β-carboxymuconic-ε-semialdehyde) may then be drawn into the NAD pathway, giving off one ^{14}C on route. A very small percentage of $^{14}CO_2$ evolved would be contributed by the NAD pathway as studies in vitro and in vivo (105) indicate. Most of the intermediate formed is oxidized stepwise by picolinic carboxylase, a dehydrogenase and a reductase, all three of which are at least 9 times as active as kynureninase, as measured in vitro (105). The reaction involving the formation of α-ketoadipic acid from α-aminomuconic acid (reductase), and the next reaction following this, cause the release of 2 $^{14}CO_2$'s from the benzene ring. The formation of $^{14}CO_2$ directly from this pathway, without involvement of other intermediary metabolic paths probably accounts, in part, for the rapid appearance of $^{14}CO_2$ within the first few hours following administration of tryptophan labeled in the benzene ring.

Four radioactive carbons remain to be accounted for. Both C_4 and $C_{7\alpha}$ become C_1 of acetate which is metabolized

earlier in the Kreb's Cycle than C_2 of acetate, derived from $C_3\alpha$ and C_5 of the benzene ring.

Therefore the rate of oxidation of T_B appears to be more rapid than that of T_2 and T_3, because 1) two of the ^{14}C's are transformed directly to $^{14}CO_2$ without entering large pools of endogenous intermediates (Kreb Cycle or C_1 intermediates); 2) of the other 4 ^{14}C's, 2 ^{14}C's are oxidized more rapidly than the ^{14}C released from tryptophan-3'-^{14}C as they form C_1 of acetate, whereas the latter is transformed to C_2 of acetate. The other two are oxidized at the same rate as the ^{14}C released from tryptophan-2- or -3'-^{14}C.

The rate of removal of the carboxyl group from tryptophan was greater than the rate of removal of ^{14}C from the other radioisomer of tryptophan (Table II). This observation can be readily explained: The carboxyl group can be cleaved (1) by deamination and subsequent decarboxylation, (2) by decarboxylation, or (3) by the pyrrolase pathway. The first two steps probably account for little of the respiratory $^{14}CO_2$ from carboxyl-labeled tryptophan if the excretion of indole-3-acetic acid derived from indolepyruvic acid or tryptamine and 5-hydroxyindoleacetic acid are used as criteria of the functioning of these pathways (24). $^{14}CO_2$ is released in the pyrrolase pathway following the action of kynureninase, alanine transaminase, and pyruvic kinase. The ^{14}C does not enter the Kreb's Cycle and hence this probably accounts for the fact that this

radioisomer appears to be metabolized at a faster rate than the other forms of radioactive tryptophan. Assuming that the other pathways for the removal of the carboxyl group are minor, it can be concluded that this form of radioactive tryptophan is a truer indicator of the activity of the pyrrolase pathway than the other forms of radioactive tryptophan. Accordingly, if T_2 and T_3, are employed, the activity of the pathway may be underestimated by 30%, with physiological doses of tryptophan (10 uM/kg).

B. Regulation of Tryptophan Metabolism

1. Enzyme Induction

L-Tryptophan

In this discussion, tryptophan-^{12}C will be used to symbolize non-radioactive tryptophan.

The rate of evolution of $^{14}CO_2$ from all radioisomers of tryptophan (10 μM/kg) was of the order of 7-12% in 6 hours. If radioactive tryptophan was administered along with a large dose of L-tryptophan (1 mmole/kg), the ^{14}C recovered in respiratory gases rose to 37%-60%, despite possible dilution of the radioisotope (Table V). In 1963 Moran and Sourkes observed the same phenomenon with DL-tryptophan-2- or -3'-^{14}C and they postulated that the increased oxidation of the radioisotope was due to the induction of tryptophan pyrrolase by L-tryptophan-^{12}C. They further surmised that the above obser-

vation suggests that physiological significance be attached to the induction of tryptophan pyrrolase by substrate (168). The present experiments are an extension of their work.

It was confirmed that the rate of oxidation of exogenous tryptophan was greatest if tryptophan-^{14}C and tryptophan-^{12}C were administered to the animal simultaneously. These results are in accord with studies in vitro: tryptophan pyrrolase activity reaches a maximum within 6 hours following treatment with tryptophan (165). The rate of oxidation of tryptophan-^{14}C decreased as the pretreatment time with tryptophan-^{12}C increased. Indeed there was no evidence of elevated tryptophan pyrrolase activity if tryptophan-^{14}C was administered 24 hours after tryptophan-^{12}C. By contrast, in adrenalectomized animals, increased tryptophan catabolism was found in 4 out of 6 animals at the end of a 24 hour period after administration of tryptophan-^{12}C. This would indicate that adrenalectomy prolongs the inductive process somewhat. The elevated rate of oxidation of tryptophan-^{14}C following a tryptophan-^{12}C load was less in adrenalectomized animals. This corresponds to the levels of tryptophan pyrrolase induced in operated animals (122). As tryptophan is a stressing agent, a component of the induction in intact animals is a consequence of a stress mechanism.

Although both DL-tryptophan-$^{14}COOH$ and the corresponding L- form were oxidized at the same rate in control rats, in

animals loaded with L-tryptophan-^{12}C, the induction of tryptophan pyrrolase was not reflected by the conversion of the DL-mixture to $^{14}CO_2$ to the same extent as the L- form. As stated earlier, the D- form contributes to respiratory $^{14}CO_2$ probably after inversion to L-tryptophan. Therefore large amounts of L-tryptophan-^{12}C and tracer amounts of D-tryptophan-^{14}C, following conversion to indolepyruvic acid, may compete for the reamination reaction. This would result in a decreased inversion of D-tryptophan-^{14}C, and, concomitantly, a reduction of respiratory $^{14}CO_2$ from this isomer. This hypothesis was proved if α-methyltryptophan was used as the inducing agent. As α-methyltryptophan is not a substrate for the transamination reaction, it would not compete with D-tryptophan-^{14}C for the enzyme. The % ^{14}C recovered from DL-tryptophan-$^{14}COOH$ following treatment with α-methyltryptophan was 15.2%; the corresponding value for L-tryptophan-$^{14}COOH$ was 14.8%. It can be inferred, therefore, that the apparent rate of catabolism of DL-tryptophan-^{14}C, in the presence of high concentrations of L-tryptophan-^{12}C, is underestimated; a truer value would be obtained by employing L-tryptophan-^{14}C as an indicator. With a loading dose of tryptophan-^{12}C 4-6 times as much tryptophan-2- or -3'-^{14}C is oxidized $\rightarrow$ $^{14}CO_2$. From the equivalence of T_2 or $T_{3'}$ oxidation, it can be concluded that kynurenine transaminase or other enzymes do not divert significant amounts of tryptophan intermediates away from the main catabolic pathway

and the increased metabolites formed do not cause any appreciable enzyme saturation. The rate of oxidation of the benzene-labeled tryptophan was somewhat lower than that of the other isomers of tryptophan if given along with a tryptophan load in adrenalectomized rats. Coupled with the fact that 3-hydroxyanthranilic acid lessened the rate of oxidation of tryptophan-benzene-^{14}C (Table VII) this strongly suggests a rate-limiting step in the total catabolic pathway. 3-Hydroxyanthranilic acid oxygenase is 86 times more active in liver than kynureninase (105). Yet Lan and Gholson have claimed that the capacity of this enzyme to oxidize its substrate *in vivo* is less than one-thousandth as great as its capacity *in vitro* (104). Although its properties *in vitro* suggest that it can become rate-limiting (see p. 17) during the catalytic process, several experiments *in vivo* lessen the validity of this hypothesis.

Hankes and Henderson (215) found that the oxidation of a large dose of 3-hydroxyanthranilic acid-$^{14}COOH$ to $^{14}CO_2$ (19.1 mg) was equivalent to that of a lesser dose (3.1 mg). *in vivo*. This would indicate that both the oxygenase and picolinic carboxylase were not rate-limiting if presented with substrate in amounts equivalent to those used in these experiments. To support this view further, Gholson *et al.* (84) found that 3-hydroxyanthranilic acid-$^{14}COOH$ was oxidized at a very rapid rate (65% in 6 hours); on the other hand,

3-hydroxyanthranilic acid-1-^{14}C was catabolized much more slowly (12% at 6 hours). A rate-limiting step, *in vivo*, beyond the level of picolinic carboxylase, dehydrogenase, and reductase could account for this discrepancy (Fig. 3).

The conversion of glutaryl CoA to acetyl CoA may be rate-limiting if the activity of this system *in vitro* corresponds to that in vivo (105). Thus the reduction in the rate of oxidation of the benzene ring of tryptophan-^{14}C, which occurs in the presence of high concentrations of tryptophan or 3-hydroxyanthranilic acid, may be due to a rate-limiting step at this level. Intermediates, beyond the level of 3-hydroxyanthranilic acid, injected along with tryptophan-benzene-^{14}C may help to localize the rate-limiting enzyme more specifically.

D-Tryptophan

D-Tryptophan was not as effective as L-tryptophan in inducing tryptophan pyrrolase, as reflected in the rate of oxidation of DL-tryptophan-3'-^{14}C. This may be a consequence of several factors, two of which stem from the transamination reaction. The high levels of D-tryptophan-^{12}C may compete for the transaminase with the ^{14}C isomer, resulting in lessened $^{14}CO_2$ production from radioactive D-tryptophan. Alternately, the induction of the enzyme is less because of a slower inversion of high concentrations of D-tryptophan to the L- form.

This explanation is tenable if it is assumed that the inductive process is proportional to the concentration of the inducing agent (168) and the induction by the D- form is possible only after its inversion to the L- form. Because tryptophan pyrrolase, measured *in vitro*, is not induced to the same extent by D-tryptophan as by the L- form (140), the latter explanation probably accounts in part for the results. On the other hand, D-tryptophan may induce the enzyme directly but less effectively than the L- isomer.

DL- α-Methyltryptophan

α-Methyltryptophan causes an increased destruction of exogenous tryptophan, as reflected by the rate of conversion of tryptophan to CO_2. Presumably this effect is related to its ability to induce tryptophan pyrrolase.

It is apparent (Fig. 13) that the induction by AMTP is a resultant of two factors, a substrate-specific effect and a stress mechanism, the latter being eliminated by adrenalectomy.

The time course of the induction by α-methyltryptophan confirms the finding of Moran and Sourkes (168); the maximum rate of tryptophan catabolism occurs 14-24 hours following administration of the amino acid analogue, and lasts as long as one week. Because of the long-lasting effect of this amino acid it was considered important to determine whether AMTP could be detected in the body during this period. These

studies (Fig. 9) indicate that the compound can be detected in serum and liver for many days after injection. Hence tryptophan pyrrolase activity may well be elevated because of the presence in the cell of the "inducing" amino acid for this period. Interestingly the maximum concentration of the amino acid analogue in liver was reached within 1-6 hours of administration, whereas tryptophan pyrrolase attained peak activity 14-24 hours later. Tryptophan is also present at maximal concentrations within 6 hours of administration but exerts its maximal effect on pyrrolase during this time period. Perhaps the initial concentration of AMTP in the liver is inhibitory; as the levels decrease the inhibition wears off, and more enzyme becomes available for the substrate.

α-Methyltryptophan could sustain a high level of pyrrolase by any combination of known mechanisms, including stimulation of de novo protein synthesis, enzyme stabilization or facilitation of combining capacity of the apoenzyme with cofactor.

It appears from this work that α-methyltryptophan is concentrated differently by various organs of the rat and most of it passes into the acid-soluble, non-protein compartment of the cell. It cannot be bound by plasma albumin as tryptophan is (7) and unlike tryptophan about 50% of the analogue is excreted unchanged within 24 hours (169); this could be the D-analogue.

One cannot decide on the basis of the present data whether α-methyltryptophan is incorporated into protein, for less than 3% of the radioactivity of liver, pancreas, and intestine, after administration of ^{3}H-α-methyltryptophan, was found in the trichloroacetic acid-precipitable substances. There is evidence that other tryptophan analogues can be incorporated into mammalian protein, including 7-azatryptophan but not β-methyltryptophan (216).

It has been suggested that the observed weight loss following injection of the compound stems from a relative deficiency of the essential amino acid tryptophan, through rapid degradation of endogenous supplies catalyzed by the induced enzyme (169). In the present work kynurenic and xanthurenic acids were measured in an effort to determine whether the metabolism of endogenous tryptophan is also affected by α-methyltryptophan, for an increased output of these compounds may reflect a high rate of catabolism of tryptophan to the stage of kynurenine and 3-hydroxykynurenine, with funneling of some of the excess of these amino acids into the quinaldic acids. From the observed increase in output of kynurenic acid and xanthurenic acid it seems reasonable to conclude that AMTP increases the rate of catabolism not only of exogenous tryptophan but of the endogenous amino acid as well.

2. Regulation of Tryptophan Metabolism by Substances Indigenous to the Rat

Corticosteroids:

Tryptophan pyrrolase activity, measured *in vitro*, is lower in adrenalectomized rats than in unoperated animals (122) under basal conditions. Presumably, adrenocorticosteroids maintain a certain level of the enzyme which falls subsequent to adrenalectomy. The drop in tryptophan pyrrolase activity was not reflected in the rate of oxidation of tryptophan *in vivo* (Table I). It can be concluded from the present investigation that the enzyme in adrenalectomized rats, although lower, is present in sufficient amount to catabolize tryptophan at a normal rate. This finding emphasizes the caution that must be taken when attempting to postulate activity of the enzyme *in vivo* from studies *in vitro*.

Copper-deficiency

The participation of copper in the action of tryptophan pyrrolase from *Pseudomonas* has been suggested by Maeno and Feigelson (128). The dependence of the enzyme activity on the availability of dietary copper was studied by placing rats on a copper-deficient diet and measuring the activity of tryptophan pyrrolase *in vitro*. No change in the enzyme activity was observed. Two conclusions are offered to account for this observation: 1) Copper is not necessary for the

activity, or 2) copper does play a role in the action of the enzyme but as was suggested by Maeno and Feigelson, it is tightly bound to the apoenzyme and not lost with a reduction in dietary copper. Or, the residual levels of copper in the liver may be sufficient to sustain the activity of the enzyme.

Metabolites of tryptophan

Certain metabolites of tryptophan, derived from several metabolic pathways are known to inhibit tryptophan pyrrolase in vitro (143, 144, 182). Thus 5-hydroxytryptophan, serotonin, and tryptamine all reduce the activity of the enzyme by 62-91% at a concentration of 10^{-3}M (143). The powerful inhibitory effect has prompted Frieden et al. to suggest that the role of serotonin as a regulator of tryptophan pyrrolase should not be overlooked. The present results indicate that serotonin, 5-hydroxytryptophan, and tryptamine have little or no influence on tryptophan oxidation in vivo, in adrenalectomized rats. Thus these metabolites probably do not inhibit pyrrolase in vivo and should not be considered of physiological importance in regulating the metabolism of tryptophan.

Metabolites of the pyrrolase pathway, including kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid have also been shown to reduce tryptophan pyrrolase activity in vitro (144). The inhibition by 3-hydroxyanthranilic acid

is competitive with tryptophan. Administration of these metabolites in high doses did not lead to alteration in the rate of oxidation of tryptophan-2- or -3'-^{14}C, both of which are acted on by pyrrolase. It can be concluded from this study that these metabolites do not exert feedback control on tryptophan pyrrolase in vivo.

Adrenergic mechanisms

Cyclic-AMP is a mediator of the metabolic response of the liver to both α - and β - adrenergic agents. Another property of cyclic-AMP is its ability to reactivate inactivated tryptophan pyrrolase in vitro (132). An attempt was made to evaluate the effect of adrenergic agents on tryptophan metabolism. Several approaches to this problem were used. The influence of adrenergic agents, adrenaline, noradrenaline, isoproterenol and dopamine, on the oxidation of tryptophan-^{14}C was studied (Table XX). At lower doses adrenaline impaired the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C, but at higher doses half of the animals metabolized tryptophan more rapidly, the rest did not. The increase of metabolism, when it occurred, was low and reached a maximum within 2 hours, falling thereafter; this is in contrast to the effect seen with a tryptophan load (Fig. 16).

Assessment of the results from this type of experiment is difficult in view of the many pharmacological actions of adrenaline in vivo. This contention was emphasized by the

finding that adrenaline lowered the rate of formate oxidation. A more direct approach to the problem was made by assaying tryptophan pyrrolase from adrenaline-treated animals. A similar experiment had been carried out by Knox in 1951 (21) but in that work tryptophan pyrrolase activity was measured several hours after the administration of adrenaline. The peak metabolic effects of this agent and noradrenaline are known to occur within 30 minutes to 1 hour of administration (217). In the present investigation, the activity of the enzyme, determined one hour following treatment with adrenaline, was not increased above control values. Thus adrenaline does not augment levels of the enzyme or alter its rate of inactivation during a period of time in which it stimulates other metabolic changes. Therefore if cyclic-AMP is increased in liver by adrenaline it does not result in any appreciable activation of tryptophan pyrrolase.

The ability of adrenalectomized rats (which were used in these studies) to synthesize cyclic-AMP has been questioned by Brodie et al. in a review of the subject (218); but in the same report evidence is presented indicating that adrenaline can still activate -- presumably via cyclic-AMP -- muscle phosphorylase in adrenalectomized rats sustained on saline.

The possibility of adrenergic regulation of tryptophan catabolism was further considered on finding that adrenergic blocking agents retard the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C. It was reasoned that if the effect was

related to adrenergic mechanisms then adrenaline should diminish the inhibition. However, this catecholamine did not have a consistent ameliorative effect on the inhibition of tryptophan catabolism.

The evidence presented indicates that adrenaline does not activate tryptophan pyrrolase in adrenalectomized rats. Assuming that cyclic-AMP is synthesized in these animals it can be concluded, albeit from indirect evidence, that this purine derivative does not play a role in the regulation of tryptophan pyrrolase in vivo.

3. Regulation of Tryptophan Metabolism by Inhibitory Drugs

A study of the effects of drugs on tryptophan oxidation in vivo indicates that many compounds reduce the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C in adrenalectomized rats. The structures of the compounds possessing this property are varied, and include tryptophan analogues, indole alkaloids and non-indole aromatic substances. The relative order of potency of these inhibitors cannot be ascertained because the pharmacological properties of certain compounds prohibit administration at comparable doses.

Of the tryptophan analogues studied, secondary and tertiary amines, including α-hydrazinotryptophan, α-hydrazino-5-hydroxytryptophan, N,N-dimethyl- and N,N-diethyl-

tryptamine, and the amide N-acetyltryptamine, were the most potent inhibitors of the formation of $^{14}CO_2$ from tryptophan-^{14}C (Table XXI). An attempt was made to establish at what phase in the formation of $^{14}CO_2$ from tryptophan-^{14}C specific compounds act. The possibility that α-hydrazinotryptophan interfered with tryptophan transport was lessened by two different types of experiment: the amount of radioactivity in the serum 15 minutes following the concurrent administration of tryptophan-3'-^{14}C and α-hydrazinotryptophan corresponded to that of controls, indicating that the initial transport of tryptophan from the peritoneal cavity into the blood was unaffected by the drug. Forty-five minutes later the radioactivity in the serum remained at high levels in the drug-treated animals but dropped in the controls (Table XIII). This would imply that either tryptophan metabolism is inhibited, resulting in a buildup of circulating tryptophan or the transport of tryptophan into certain organs is diminished. Measurement of tryptophan-^{14}C in liver and other organs would help to clarify this point. That tryptophan transport was probably not responsible for the reduction in expired $^{14}CO_2$ was emphasized by a comparison of the effects of the hydrazino analogue on the oxidation of three forms of radioactive tryptophan. The analogue retarded the formation of respiratory $^{14}CO_2$ from tryptophan-2- or -3'-^{14}C but not from benzene-labeled tryptophan (Table XII). If the transport of tryptophan was impeded one would expect all three forms of

radioactive tryptophan to be reduced equally. While the above does not definitely rule out transport as a site of action of the compound it does, however, indicate that another effect may exist, possibly on tryptophan catabolism.

According to the accepted pathway for the catabolism of tryptophan the removal of ^{14}C from tryptophan-2-^{14}C precedes the removal of ^{14}C from tryptophan-3'-^{14}C or tryptophan-benzene-^{14}C. Therefore the level of inhibition of tryptophan-2-^{14}C oxidation would be expected to establish the level for the other isomers of tryptophan-^{14}C. Yet α-hydrazinotryptophan reduced the oxidation of tryptophan-3'-, -2-, benzene-^{14}C in decreasing order of magnitude (Table XII). Because tryptophan-3'-^{14}C was inhibited to a greater extent than tryptophan-2-^{14}C, it can be concluded that α-hydrazinotryptophan has alternate sites of action, but not involving a differential effect of alanine or formate oxidation because both were only slightly inhibited and to the same extent. Hydrazine derivatives are known to inhibit B_6-dependent enzymes by trapping the carbonyl group of pyridoxal phosphate. The removal of ^{14}C from T3 is performed by the B_6-enzyme kynureninase. The activity of this enzyme is lowered <u>in vitro</u> in the presence of hydrazine or phenylhydrazine (62). Therefore one possible site of action of α-hydrazinotryptophan is at the level of kynureninase.

α-Hydrazino-5-hydroxytryptophan and α-methyl, α-

hydrazino-3, 4-dihydroxyphenylpropionic acid (MK-485) exhibited the same differential effect on the two forms of radioactive tryptophan. The structural analogy between MK-485 and 3-hydroxykynurenine, should not be overlooked. It may be of interest to ascertain whether other hydrazine derivatives, usually employed as monoamine oxidase inhibitors, exert an effect on tryptophan catabolism.

The lack of inhibition of the metabolism of the benzene-labeled tryptophan by α-hydrazinotryptophan (Fig. 17) is difficult to explain on the basis of the accepted order for the stepwise oxidation of the carbons from tryptophan. The drug may inhibit the oxidation of formate and alanine formed from T_2 and T_3, and exert no influence on tryptophan catabolism. This hypothesis is made less likely by the finding that the oxidation of exogenous alanine and formate are inhibited to a minor extent by the drug. And, theoretically, the tryptophan analogue is more likely to inhibit the catabolism of tryptophan than the catabolism of both alanine and formate.

Or, alternatively, another pathway for the total oxidation of the benzene ring of tryptophan is conceivable which does not involve cleavage of the pyrrole ring or alanine side chain as the initial steps, and which is unaffected by α-hydrazinotryptophan. This may exist either in rat tissue or in the intestinal flora. As stated in the "Introduction" there is some evidence pointing to this possibility. Further

investigations, especially isolation of the intermediates formed from tryptophan labeled in the benzene ring, may help to clarify this.

Other tryptophan analogues which lowered the formation of respiratory $^{14}CO_2$ from labeled tryptophan include 5- and 6-methyltryptophan, both of which cause a slight reduction in tryptophan pyrrolase activity following administration (130), and inhibit tryptophan pyrrolase *in vitro* (143), hypaphorine, a naturally-occurring tryptophan analogue, and 4-hydroxytryptophan, α-methyl-5-hydroxytryptophan, an inhibitor of the serotonin and tryptamine pathways (178). α-Substituted, and N-substituted derivatives of tryptamine, which are antagonists of serotonin metabolism (180) retarded the rate of appearance of $^{14}CO_2$ in respiratory gases from tryptophan-^{14}C. Some of these compounds including α-methyltryptamine, and N,N-dimethyltryptamine are metabolized by 6-hydroxylation (32), and thus may exert their effect following conversion.

In conclusion several analogues of tryptophan, notably α-hydrazinotryptophan have been identified as inhibitors of tryptophan-^{14}C oxidation *in vivo*. A specific effect of α-hydrazinotryptophan on the catabolic pathway was suggested by two different experimental approaches. Indirect effects by the other compounds cannot be ruled out by the present type of investigation, and additional studies using other

methods, such as measurement of urinary metabolites of the pyrrolase pathway, may help to further localize the site of action. The present investigation indicates that serotonin antimetabolites may exert an effect on yet another area of tryptophan metabolism, that of the catabolic pathway of tryptophan.

Although many indoles did influence tryptophan catabolism, others did not, suggesting that the indole nucleus alone is not sufficient to satisfy all the structural requirements necessary to render it an antimetabolite of tryptophan. This point was further emphasized by a study of the effects of indole alkaloids on tryptophan oxidation (Table XIV). Certain alkaloids including yohimbine, its geometric isomer, corynanthine, harmaline and noribogaine reduced the formation of respiratory $^{14}CO_2$ from tryptophan-2- or -3'-^{14}C but others (dihydroergotamine and L.S.D.) were ineffective. Most of these alkaloids including harmaline and yohimbine have pharmacological actions which could account for the observed reduction in tryptophan oxidation, including depression of blood pressure, temperature or respiration. Formate oxidation (Table XVII) was unaffected by yohimbine and harmaline implying that non-specific effects on respiration or temperature were unrelated to the observed depression of tryptophan oxidation. The lack of effect of yohimbine or harmaline on the oxidation of benzene-labeled tryptophan, and the increased radioactivity in liver following simultaneous administration

of both tryptophan-^{14}C and either of these alkaloids with respect to controls, gave credence to the view that tryptophan transport was not impeded. However definite conclusions concerning the effect of these compounds on tryptophan transport await the measurement of tryptophan specifically. Harmaline which was more effective on the side chain than on the pyrrole-labeled tryptophan (Table XV) inhibited exogenous alanine oxidation considerably suggesting that its greater effect on tryptophan-3'-^{14}C was a consequence of its action on alanine oxidation.

Corynanthine was effective at 20 times the dose of yohimbine yet it is a more active adrenergic blocking agent than yohimbine (186) implying that there is no parallel relationship between adrenergic blockade and inhibition of tryptophan catabolism by these alkaloids. Ibogaine (Fig. 4) which has a similar action on the central nervous system to that of yohimbine (219) was a weak inhibitor of the metabolism of tryptophan whereas noribogaine, reduced the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C considerably. The iboga alkaloids have hypotensive effects (220) which could account for the observed action of noribogaine. But the weak effect of ibogaine diminishes the plausibility of this explanation.

The inconsistent effects of ergotamine may be explained on the basis of its multiple pharmacological actions to which

this method of measuring tryptophan metabolism is sensitive. Maffei-Faccioli found that the induction of pyrrolase by reserpine is unimpaired in the presence of ergotamine (199) suggesting that ergotamine exerts no influence on tryptophan catabolism at the level of pyrrolase.

Another ergot alkaloid, L.S.D., did not inhibit tryptophan catabolism, in fact, it stimulated slightly the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C. Nomura observed that administered L.S.D. causes a slight, but not significant increase in tryptophan pyrrolase activity (221), which may account for the observed effects in vivo. However, as intact animals were used in those experiments, the increase in pyrrolase activity may be a consequence of a stress induction.

In conclusion, several indole alkaloids of diverse structure antagonize tryptophan degradation in vivo but the site of action has not been determined with certainty. Certain members of this group of alkaloids, known antagonists of serotonin metabolism (yohimbine and harmaline), can be said to reduce the formation of $^{14}CO_2$ derived from tryptophan-^{14}C, by its catabolic route. The hypothesis advanced by Shaw and Woolley, that these alkaloids are antimetabolites of serotonin by virtue of their indolic nucleus cannot be extended to an antimetabolite hypothesis of tryptophan catabolism. Some, but not all, indole alkaloids inhibited the catabolism of tryptophan.

As yohimbine and corynanthine are adrenergic blocking drugs (186) it was considered of interest to extend further the study of the effects of adrenergic blocking agents on tryptophan metabolism. Certain α - and β - adrenergic blocking drugs, structurally unrelated to tryptophan, diminished the conversion of tryptophan-$^{14}C \longrightarrow {}^{14}CO_2$ (Table XXII, XXIII), whether given at the same time or 1 hour prior to tryptophan-^{14}C. Dibenzyline, an α -blocking agent exerted a long-lasting effect in accord with its prolonged pharmacological actions (186). The high pharmacological and chemical reactivity of this drug (189) and its virtual absence in the liver within 2 hours of administration (191), made it obligatory to determine whether it was acting non-specifically. The formation of respiratory $^{14}CO_2$ from both alanine and formate was reduced by dibenzyline implying that the observed reduction in tryptophan catabolism may very well be a non-specific effect. The other α -blocker, phentolamine, which diminished the appearance of respiratory $^{14}CO_2$ from tryptophan-^{14}C had no influence on alanine oxidation (Table XXIV), implying that its effect is by a mechanism unrelated to non-specific causes, such as on respiration.

All three β -adrenergic blocking agents, including pronethalol, propranolol, and dichloroisoproterenol lowered the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C (Table XXIII). This could not be explained on the basis of

a non-specific action such as on respiration as both pronethalol and DCI did not reduce the rate of oxidation of alanine or formate (Table XIV). An attempt was made to ascertain whether pronethalol and DCI had any influence on the activity and properties of tryptophan pyrrolase, measured in vitro. Pronethalol was without effect whereas DCI lowered the activity of the enzyme which also was inactivated more rapidly. Conclusions from this type of experiment must be guarded because 1) homogenization of tissue could bring the drugs into contact with the enzyme, a process that may not occur in vivo, and 2) the enzyme-drug complex, if formed in vivo, may be dissociated by the process of dilution.

It is difficult to assess the relationship between the known pharmacological actions of these drugs, that is, their adrenergic blocking action and their effect on tryptophan catabolism. A parallel relationship between these effects is unlikely for several reasons: 1) Corynanthine and propranolol are more active adrenergic blocking agents than yohimbine and pronethalol, respectively (186, 198). Yet the latter compounds were more active as inhibitors of tryptophan catabolism. 2) Some but not all α-adrenergic blocking agents tested exerted an influence on the rate of oxidation of tryptophan. One would expect a uniform effect by all the α-blockers if a similar mechanism is responsible for this action. 3) The adrenergic blocking effect of propranolol and pronethalol is very short, the half-life being in the order of 35-60 minutes

yet these compounds lessened the formation of $^{14}CO_2$ from tryptophan-^{14}C for at least 7 hours. 4) Adrenaline did not consistently relieve the action of these compounds.

The mechanism by which these agents reduce the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C remains to be revealed by other methods. If the action of these compounds is on the pyrrolase pathway of tryptophan, examination of the levels of urinary metabolites of tryptophan and of the concentration of tryptophan in several organs may help to locate the site of action of these compounds.

C. Inhibition of Tryptophan Pyrrolase

If enzyme inhibition is responsible for the observed effects of the drugs on tryptophan catabolism, it is conceivable that tryptophan pyrrolase could be one of the target enzymes. The effects of these drugs on tryptophan pyrrolase *in vitro* is determined in an attempt to correlate their action on both systems.

The present investigation indicates that the enzyme is inhibited by the same compounds which reduce tryptophan oxidation *in vivo*; a few exceptions are to be noted.

Tryptophan Analogues

Of the tryptophan analogues, tryptophol was the most potent inhibitor of the enzyme (Fig. 4, Table XXVIII). The

non-competitive type of inhibition by this analogue gives it the distinction of being the first identified "side-chain variant" of tryptophan which inhibits in this manner. Frieden et al. found that all "side-chain variants" were competitive antagonists (143). The extent of inhibition, if tryptophol was in contact with the enzyme for 5 minutes, or a further 25 minutes, remained the same, implying a rapid formation of the enzyme-inhibitor complex or an irreversible inactivation of the enzyme. The latter possibility is made less likely by the finding that tryptophol protects the enzyme against heat inactivation (130).

The recognition that tryptophol is an inhibitor of tryptophan metabolism in microorganisms (222) can now be extended to include inhibition of tryptophan metabolism in mammalian tissue.

α-Hydrazinotryptophan was a more effective inhibitor of the enzyme than tryptophol, but only at low concentrations (Fig. 22). The reversal of the degree of inhibition at higher concentrations may imply that the type of inhibition exerted by these compounds is dependent on their concentration.

The 5-hydroxy analogues of 2 tryptophan derivatives exhibited a different effect on the enzyme than their parent compounds: α-methyl-5-hydroxytryptophan inhibited the enzyme at 10^{-3}M conc. whereas α-methyltryptophan did not. Conversely the 5-hydroxy analogue of α-hydrazinotryptophan was a weaker

inhibitor than α-hydrazinotryptophan at equimolar concentrations; this would imply that ring-substituents influence the formation of the enzyme-inhibitor complex. These results support the findings of Frieden et al., that ring-substituted derivatives of tryptophan are non-competitive antagonists of the enzyme, as opposed to "side-chain variants" (143). It is also of interest to note that α-methyl-5-hydroxytryptophan is a more potent inhibitor than its parent compound, of another tryptophan-degrading enzyme, aromatic amino acid decarboxylase (178).

Many of the compounds studied in vitro cause a further loss in activity of the enzyme if preincubated with it in the absence of substrate. Primary amines, notably tryptamine, react more extensively than secondary amines implying that the affinity of the former compounds for the enzyme is greater, or that the latter protect the enzyme against inactivation. On the other hand, the inhibition developed by indole-3-acetic acid was eliminated by preincubation. This compound is known to protect the enzyme considerably against inactivation (141); thus the activity of the enzyme following incubation with the acid is a resultant of two opposing mechanisms. It would be important to study the effects of these compounds on the fully activated enzyme (122).

Adrenergic Blocking Agents

The α-adrenergic blocking agents (dibenzyline, dibena-

mine, phentolamine) inhibit tryptophan pyrrolase, with the exception of ergotamine and its dihydro derivative, used at one-tenth the concentration of the others. None of the β-adrenergic agents exerted an influence on pyrrolase except under very favourable reaction conditions, i.e. with preincubation or with a concentrated preparation of the enzyme. These observations, combined with the fact that adrenaline is a very potent inhibitor of the enzyme in vitro (143) would suggest that the enzyme is susceptible to inhibition by α-adrenergic blocking drugs in a manner analogous to the α-receptor. For α-receptor complex formation, a small cationic head as quarternary ammonium is essential (223) and bulky constituents such as possessed by the β-adrenergic blocking agents, prevent the interaction.

Evidently the analogy cannot be adhered to rigidly as not all the adrenergic blocking agents (yohimbine) act on the enzyme in a similar manner, and the degree of blocking action by yohimbine and corynanthine is inversely related to their degree of inhibition of the enzyme (186).

The adrenergic blocking drugs are known to interfere with lipid and carbohydrate metabolism. These results show that they may also alter amino acid metabolism, at least in vitro.

Comparison of *In Vivo* - *In Vitro* Data

The studies *in vitro* lend support to the possibility that reduction of tryptophan-^{14}C oxidation following administration of tryptophan analogues or indole alkaloids may reflect an inhibition of tryptophan pyrrolase *in vivo*.(Table XXXII). All the indoles which lowered the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C inhibited tryptophan pyrrolase *in vitro*. Only ibogaine in this group inhibited tryptophan oxidation slightly *in vivo* yet had no effect on tryptophan pyrrolase *in vitro* (Table XIV). Some compounds that are ineffective or exerted inconsistent effects *in vivo*, including tryptamine, 5-hydroxytryptophan and tryptophol, are potent inhibitors of the enzyme. As these compounds are indigenous to the rat, they may be metabolized within shorter periods of time than the synthetic compounds and thus exert only a transitory effect *in vivo*.

It is of interest to inquire whether the doses at which certain compounds were administered could achieve inhibition of the enzyme as they do *in vitro*. If only 4% of the injected dose of α-hydrazinotryptophan is accumulated by the liver this amount would be sufficient to account for the reduction in the catabolism of tryptophan, *in vivo*, on the basis of inhibition of tryptophan pyrrolase. This figure is calculated from the dose-response curve.

The present investigation indicates that administration

Table XXXII. A comparison of the effect of indoles on tryptophan-^{14}C oxidation *in vivo* and on tryptophan pyrrolase activity *in vitro*.

The dose of the administered drug is 50 mg/kg, *in vivo* and the % inhibition at 2 hours is used; the concentration of the drug *in vitro* is 10^{-3}M. Please see Tables X, XI, XIV, XXVIII, XXIX, for further description. % inhibition is denoted as follows: 1-25% (+), 25-50% (++), 50-100% (+++).

COMPOUND	% INHIBITION	
	IN VIVO	IN VITRO
α-Hydrazinotryptophan	+++	+++
Hypaphorine	+	+
Tryptophol	Inconsistent	+++
α-Methyl-5-hydroxytry.	+++	++
4-Hydroxytryptophan	+	+++
α-Hydrazino-5-hydroxytry.	+++	+++
Tryptamine	0	++
α-Methyltryptamine	+	+
α-Ethyltryptamine	++	+
N-Acetyltryptamine	+++	+++
N,N-Diethyltryptamine	++	+
Ibogaine[a]	+	0
Ibogamine[a]	0	0
Noribogaine[a]	+++	++
Yohimbine[b]	++	++(+)
Corynanthine [c]	++(+)	+

a 40 mg/kg

b 5 mg/kg

c 100 mg/kg

of indolic substances may achieve inhibition of the pyrrolase pathway. The potential alteration of this pathway could very well influence the extent of diversion of tryptophan to the serotonin or tryptamine pathways. It would be important, therefore, to determine the rate of conversion of tryptophan to kynurenine when studying antagonists of serotonin metabolism.

Whether inhibition of tryptophan pyrrolase does account for the observed reduction in tryptophan oxidation by the adrenergic blocking agents cannot be decided on the basis of the present investigation. If the α-adrenergic blocking agents inhibit tryptophan pyrrolase by "irreversible" alkylation one would expect a reduction in the activity of the hepatic enzyme from drug-treated animals. Yet administration of dibenzyline did not lead to lowered enzyme activity. Additionally, Maffei-Faccioli et al. found no reduction in pyrrolase following administration of either dibenamine or phentolamine (199).

It is quite unlikely that the β-adrenergic blocking agents reduce the formation of respiratory $^{14}CO_2$ from labeled tryptophan by inhibition of pyrrolase. The activity of the enzyme was unaffected by high concentrations of the drug; furthermore, very little of either propranolol or pronethalol can be detected in hepatic tissue within 2 hours of administration (198).

It is conceivable that these compounds still cause an apparent reduction in the activity of the enzyme by altering the environment of the enzyme *in vivo*, for example, by reducing hepatic blood flow. It must be noted that these experiments were performed in adrenalectomized rats. An entirely different effect may be observed in intact animals.

IV. SUMMARY

Tryptophan catabolism, and its regulation by enzyme induction, by substances indigenous to the rat, and by drugs was studied.

Physiological doses of tryptophan-2-^{14}C, tryptophan-3'-^{14}C, and tryptophan-benzene-u-^{14}C are oxidized fully to $^{14}CO_2$ in vivo at similar rates. A rate-limiting step at the level of 3-hydroxyanthranilic acid oxygenase, or beyond is conceivable as the rate of oxidation of benzene-labeled tryptophan is reduced if administered along with high doses of 3-hydroxyanthranilic acid.

The rate of catabolism of tryptophan-^{14}C in vivo can be increased by administration of a load of L-tryptophan, DL-α-methyltryptophan, or D-tryptophan, presumably by induction of tryptophan pyrrolase. Also, an increased rate in the catabolism of endogenous tryptophan, as measured by the urinary excretion of kynurenic and xanthurenic acids, is observed in the rats administered with α-methyltryptophan.

The time course of the induction of the enzyme, as reflected in the rate of conversion of tryptophan-^{14}C to $^{14}CO_2$, was traced by a method of staggered injections of tryptophan-^{12}C and tryptophan-^{14}C. It was confirmed that the response of the enzyme to tryptophan wears off within 24 hours, but the response to α-methyltryptophan persists for as long as one week. Moreover the maximum rate of tryptophan catabolism

is seen 14-24 hours following administration of the amino acid analogue, as opposed to 6 hours with the parent compound.

As the time course of the induction by α-methyltryptophan did not correspond to that of tryptophan, the distribution of α-methyltryptophan in the body was studied in an attempt to correlate its concentration in various tissues with its effect on pyrrolase. α-Methyltryptophan was accumulated by several organs, including kidney, pancreas and liver and was detectable therein for many days, indicating that tryptophan pyrrolase levels may well be elevated because of the presence in the cell of the inducing agent during this period. The maximum levels of this amino acid in the liver were attained 1-6 hours following administration, indicating that the maximum response of the enzyme to α-methyltryptophan does not correspond to the maximum levels of the amino acid in hepatic tissue.

Another portion of this investigation was devoted to an examination of the hypothesis that certain metabolites of tryptophan shown to depress the activity of tryptophan pyrrolase, may function similarly in vivo. If such were the case, presumably these compounds would serve as regulators of the rate of degradation of tryptophan. However, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, serotonin, 5-hydroxytryptophan did not influence the rate of catabolism of tryptophan-3'-^{14}C in vivo to any significant extent.

The rate of conversion of tryptophan-^{14}C to $^{14}CO_2$ was

found to be lowered by drugs of diverse structure, including tryptophan analogues, indole alkaloids, and adrenergic blocking drugs.

α-Hydrazinotryptophan, α-hydrazino-5-hydroxytryptophan diminish the formation of respiratory $^{14}CO_2$ from tryptophan-3'-^{14}C and to a lesser extent, tryptophan-2-^{14}C. This would indicate that both drugs have several sites of action, perhaps on kynureninase, a B_6-dependent enzyme which is responsible for the removal of the ^{14}C from T_3.

Both these compounds inhibit tryptophan pyrrolase *in vitro* indicating an effect which may account for their action on tryptophan catabolism *in vivo*. Further support for reduced metabolism stemming from enzyme inhibition was provided by the correlation between inhibition of tryptophan catabolism *in vivo* and the elevated levels of radioactivity in serum following administration of α-hydrazinotryptophan.

Certain tryptophan analogues which are ineffective *in vivo* including tryptophol and tryptamine are potent inhibitors of the enzyme *in vitro*.

Yohimbine, corynanthine, noribogaine all reduce the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C and inhibit tryptophan pyrrolase *in vitro*. Yohimbine lowers the activity of the enzyme by preventing combination of hematin with the enzyme.

Some of the compounds which inhibit tryptophan oxidation

either *in vivo* or *in vitro* are known antagonists of serotonin metabolism. The present investigation indicates that possible effects of the drugs on the catabolism of tryptophan *in vivo* should be taken into consideration when studying antimetabolites of serotonin.

Both α- (dibenzyline, phentolamine) and β - (pronethalol, propranolol, dichloroisoproterenol) adrenergic blocking agents diminish the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C, but their site of action has not been determined with certainty. Phentolamine, dibenzyline and dibenamine inhibit tryptophan pyrrolase *in vitro*, the latter two compounds presumably acting as alkylating agents. Administration of dibenzyline, however, does not lead to a reduction in tryptophan pyrrolase activity. The β-adrenergic blocking agents inhibit pyrrolase only under very favourable reaction conditions, and it is therefore unlikely that their observed effect *in vivo* stems from inhibition of tryptophan pyrrolase. The present investigation indicates that these blocking agents, known to alter the metabolism of carbohydrates and lipids, influence amino acid metabolism as well, *in vitro*, and possibly *in vivo*.

V. CLAIMS TO ORIGINAL CONTRIBUTION TO KNOWLEDGE

1. The rate of formation of respiratory $^{14}CO_2$ from tryptophan uniformly-labeled with ^{14}C in the benzene ring is reduced if 3-hydroxyanthranilic acid is administered concurrently with the radioactive material. A rate-limiting enzyme in the catabolic pathway could account for this effect.

2. An increased catabolism of endogenous tryptophan, determined by measurement of the tryptophan metabolites, kynurenic and xanthurenic acids, is observed following administration of α-methyltryptophan. Presumably this effect is a result of the induction of tryptophan pyrrolase by the α-methyl analogue.

3. Studies on the distribution of DL-α-methyltryptophan indicate that it is accumulated by many organs including kidneys, pancreas, liver and brain. Peak levels of this compound in the liver are attained within 6 hours following administration and therefore do not correspond to the maximal increase in the rate of tryptophan catabolism observed 14-24 hours later. The compound can be detected in the serum and several organs for many days corresponding in time with the prolonged induction of tryptophan pyrrolase by this compound.

4. D-Tryptophan causes an increased catabolism of exogenous DL-tryptophan-^{14}C presumably by induction of tryptophan pyrrolase, either directly or after inversion to the L- isomer.

5. The oxidation of physiological doses of tryptophan-3'-^{14}C is diminished in vitamin B_6-deficient rats. By a process of elimination, a reduction in the activity of kynureninase or another B_6-dependent enzyme in the catabolic pathway was considered to be responsible for this effect.

6. α-Hydrazinotryptophan, and its 5-hydroxy analogue, reduced the formation of $^{14}CO_2$ from tryptophan labeled with ^{14}C in the side chain and to a lesser extent pyrrole ring-labeled tryptophan. It is thought that both compounds have more than one site of action on tryptophan catabolism. α-Hydrazinotryptophan has no influence on the rate of catabolism of tryptophan-benzene-^{14}C.

7. Yohimbine, corynanthine, harmaline and noribogaine, all indole alkaloids, reduce the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C. Yohimbine and harmaline exert this effect on either tryptophan-2- or 3'-^{14}C but have little influence on benzene-labeled tryptophan.

8. Dibenzyline, phentolamine, pronethalol, propranolol and dichloroisoproterenol, all adrenergic blocking agents, lessen the formation of respiratory $^{14}CO_2$ from tryptophan-^{14}C. None of these compounds have an appreciable inhibitory effect on alanine or formate oxidation, with the exception of dibenzyline, indicating an action unrelated to non-specific causes, such as on respiration.

9. Certain properties of two forms of tryptophan pyrrolase, apoenzyme combined with hematin and endogenous holoenzyme, are different, in vitro. (1) The apoenzyme-hematin complex is spontaneously inactivated to a lesser extent than endogenous holoenzyme. (2) α-Methyltryptophan protects the latter form to a greater degree than the apoenzyme-hematin complex against spontaneous inactivation. (3) α-Hydrazinotryptophan inhibits the apoenzyme-hematin complex to a greater extent than the endogenous holoenzyme. It is suggested that hematin may not be identical with the endogenous cofactor.

10. Tryptophol, N-acetyltryptamine, α-hydrazinotryptophan, 4-hydroxytryptophan, α-hydrazino-5-hydroxytryptophan, and α-methyl-5-hydroxytryptophan all inhibit the activity of tryptophan pyrrolase in vitro, in decreasing order of effectiveness. α-Hydrazinotryptophan and tryptophol do so non-competitively.

11. Yohimbine, corynanthine and noribogaine inhibit tryptophan pyrrolase *in vitro*. Yohimbine does so non-competitively, and appears to act by interfering with the combination of enzyme and hematin.

12. Dibenamine, dibenzyline and phentolamine lower tryptophan pyrrolase activity *in vitro*; the first two substances are non-competitive antagonists and are more potent than the latter compound. Pronethalol, propranolol, and dichloroisoproterenol lower the activity of the enzyme only if preincubated with it in the absence of substrate.

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