Running Title: Transport of Tryptophan Into Cerebral Cortex Slices

THE TRANSPORT OF L-TRYPTOPHAN INTO SLICES OF RAT CEREBRAL CORTEX

by

Maureen E. Kiely

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

McGill University
Montreal, Quebec

Department of Biochemistry
May, 1974

For my parents

ABSTRACT

THE TRANSPORT OF L-TRYPTOPHAN INTO SLICES OF RAT CEREBRAL CORTEX

MAUREEN E. KIELY

Ph.D. Biochemistry

Rat cerebral cortex slices incubated aerobically at 37°C in Krebs-Ringer phosphate medium containing 10 mM glucose and 1.0 mM L-tryptophan-{carboxyl-^{14}C} accumulated L-tryptophan. Uptake of L-tryptophan was linear for the first 15-20 min. Maximum L-tryptophan concentration was attained within 60 min. The transport mechanism became saturated with 1-3 mM L-tryptophan in the medium. Entry of the amino acid into the cells was thereafter directly proportional to its initial concentration in the medium.

L-Tryptophan uptake decreased significantly under the following experimental conditions: lowering the incubation temperature to 4°C and/or incubating under N_2, omitting glucose, decreasing the Na^+ concentration below 25 mM or omitting K^+ from the medium. These results indicated that accumulation of L-tryptophan against its concentration gradient is an active process.

The effects of a number of amino acids, tryptophan metabolites and tryptophan analogs on the uptake of L-tryptophan were studied. L-Phenylalanine, D,L-p-chlorophenylalanine, L-tyrosine, L-5-hydroxytryptophan, L-DOPA, L-leucine, L-isoleucine, L-valine, and L-kynurenine inhibited tryptophan transport. It was concluded that L-tryptophan enters the cortex cell via the large neutral amino acid carrier system.

In vivo alterations to the physiological state of the rats e.g., prolonged dietary deficiency of tryptophan, adrenalectomy or hypophysectomy, had no effect on L-tryptophan accumulation by cortex slices from the brains of these animals.

RESUME

LE TRANSPORT DU L-TRYPTOPHANE DANS LES TRANCHES DE CORTEX CÉRÉBRAL DE RAT

Maureen E. Kiely

Ph.D. Biochimie

Les tranches de cortex cérébral de rat, incubées en presence d'oxygène à 37°C, dans un milieu physiologique Krebs-Ringer phosphate contenant du glucose, 10 mM, et du L-tryptophane-^{1}COOH, 1 mM, accumulèrent le L-tryptophane. La capture du L-tryptophane fut linéaire durant les quinze à vingt premières minutes. La concentration maximale de L-tryptophane fut atteinte en dedans de 60 minutes. Avec des concentrations de 1 à 3 mM de L-tryptophane dans le milieu, les mécanismes de transport atteignirent un point de saturation. A partir de ce moment, l'entrée de l'acide aminé dans les cellules fut directement proportionnelle à sa concentration initiale dans le milieu d'incubation.

La capture du L-tryptophane a diminué d'une façon significative dans les conditions expérimentales suivantes: abaissement de la température d'incubation à 4°C, et/ou incubation dans une atmosphère azotée, manque de glucose, diminution de la concentration de Na^+ au-dessous de 25 mM ou omission du K^+ dans le milieu. Les résultats indiquèrent que l'accumulation du L-tryptophane contre son gradient de concentration est un phénomène actif.

Les effets d'un certain nombre d'acides aminés, de métabolites du tryptophane, et de composés de structure analogue au tryptophane sur la capture du L-tryptophane furent étudiés. La L-phenylalanine, D,L-p-chlorophenylalanine, L-tyrosine, le L-5-hydroxytryptophane, la L-DOPA, L-leucine, L-isoleucine, L-valine et L-kynurenine inhibèrent le transport du L-tryptophane. Nous avons donc conclu que le L-tryptophane pénètre dans les cellules corticales par le système de transport réservé aux acidés aminés neutres de poids moléculaire plus élevé.

Des modifications faites *in vivo* sur l'état physiologique des rats telles que, une absence prolongée de tryptophane dans la diète, une adrénalectomie, ou une hypophysectomie, ont aucun effet sur l'accumulation du L-tryptophane par les coupes du cortex cérébral de ces animaux.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my research director, Dr. Theodore L. Sourkes for his generous assistance and valuable guidance throughout the course of this investigation.

I would like to thank Dr. Simon Young for his helpful suggestions during the preparation of this manuscript.

My thanks are extended to Dr. Ann Kato and Mr. Francois Feldmüller for proof-reading this thesis.

I am indebted to Mrs. Ellen Morton, Mrs. Maria Naccarato and Mrs. Tanya Rosenberg for typing the thesis, to Dr. Linda Bloomer and Miss E. Swezey for drawing the figures in the text and to Mr. Levon Guluzian for photographing these figures.

Heartfelt thanks to my husband Dr. Samarthji Lal, my "nearest neighbor" in the laboratory during the course of the investigations presented here and my understanding fiance throughout the preparation of this manuscript.

This work was supported by a grant to Dr. T.L. Sourkes from the Medical Research Council of Canada.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

adrex	adrenalectomy
ATP	adenosine triphosphate
detns	determinations
2,4-DNP	2,4-dinitrophenol
exptl.	experimental
FAD	flavin adenine dinucleotide
GABA	γ-aminobutyric acid
hypox	hypophysectomy
L-DOPA	L-3,4-dihydroxyphenylalanine
K_m	Michaelis constant
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
S.E.	standard error
V_{max}	maximal velocity

PREFACE

The study of tryptophan transport into rat cerebral cortex slices which is presented to the reader on the succeeding pages of this thesis forms a small segment of a continuing research program on tryptophan metabolism in brain. This program, begun about twenty years ago, is under the direction of Dr. Theodore L. Sourkes, Laboratory of Chemical Neurobiology, Departments of Biochemistry and Psychiatry, McGill University.

At the outset of this study, Sourkes, Missala and Oravec (1970) had just shown that the tryptophan analogue, α-methyltryptophan, decreases the concentrations of brain serotonin and 5-hydroxyindoleacetic acid. They had postulated that these decreases were caused by a deficient supply of the precursor tryptophan.

At the same time, a number of investigators were actively engaged in studies of the rate-controlling step in the serotonin pathway of tryptophan metabolism. Several reports had indicated that tryptophan-5-hydroxylase (EC 1.14.3.3), the first enzyme in this pathway, is the rate-limiting step (Eccleston, Ashcroft and Crawford, 1965; Jequier, Lovenberg, and Sjoerdsma, 1967; Moir and Eccleston, 1968). However, since the Michaelis constant for this enzyme was initially found to be much higher

than the normal level of brain tryptophan, it was concluded that tryptophan-5-hydroxylase is unsaturated under physiological conditions (Jequier, Robinson, Lovenberg and Sjoerdsma, 1969). Thus the question arose: could the transport of tryptophan into brain cells be a significant factor in serotonin biosynthesis? Finally, although extensive research had already been done on the transport of some amino acids into brain (Neame, 1968), little was known concerning the mode of entry of tryptophan into brain cells nor of the factors which might facilitate or inhibit this entry. Thus it was with the above studies in mind that this project was undertaken.

INTRODUCTION

1. Tryptophan - An Essential Amino Acid

Tryptophan, a nutritionally indispensable amino acid was first isolated by Hopkins and Cole (1901) from a tryptic digestion of casein. The correct structure, α-amino-β-indole-propionic acid, was proposed by Ellinger (1906) and was later confirmed by Ellinger and Flamand (1907) using chemical synthesis. The necessity of this amino acid for growth was demonstrated initially by Willcock and Hopkins (1906). Since then numerous nutritional studies have confirmed that tryptophan is absolutely essential for the growth and development of mammalian species (Abderhalden, 1912; Mendel, 1914-15; Rose, 1938). Research with bacterial mutants of Escherichia coli, Neurospora crassa and Aerobacter aerogenes, however, has shown that microorganisms are capable of synthesizing tryptophan (Meister, 1965). The possibility of a biosynthetic pathway for tryptophan formation by plants has, to date, received little investigation. It is known, however, that the plant growth hormone, indoleacetic acid, is derived from tryptophan but it has not yet been determined whether this tryptophan is a product of plant cell synthesis or whether it is an essential nutrient (Meister, 1965).

2. Metabolism of Tryptophan

Because this study is concerned with the transport of tryptophan into slices of rat cerebral cortex and because some tryptophan metabolites may help to control the entry of this amino acid into brain, a brief review of tryptophan metabolism as related to mammalian cells will be presented here. To date, four routes for tryptophan utilization have been elucidated:

(A) the partial metabolic destruction of tryptophan to carbon dioxide, water and ammonia resulting in the formation of the vitamin niacin as well as numerous metabolites (Figure 1),

(B) the hydroxylation and decarboxylation of tryptophan to yield the potent vasoconstrictor, serotonin (Figure 2),

(C) deamination and transamination, routes whereby the D isomer can be used by the cell (Figure 2),

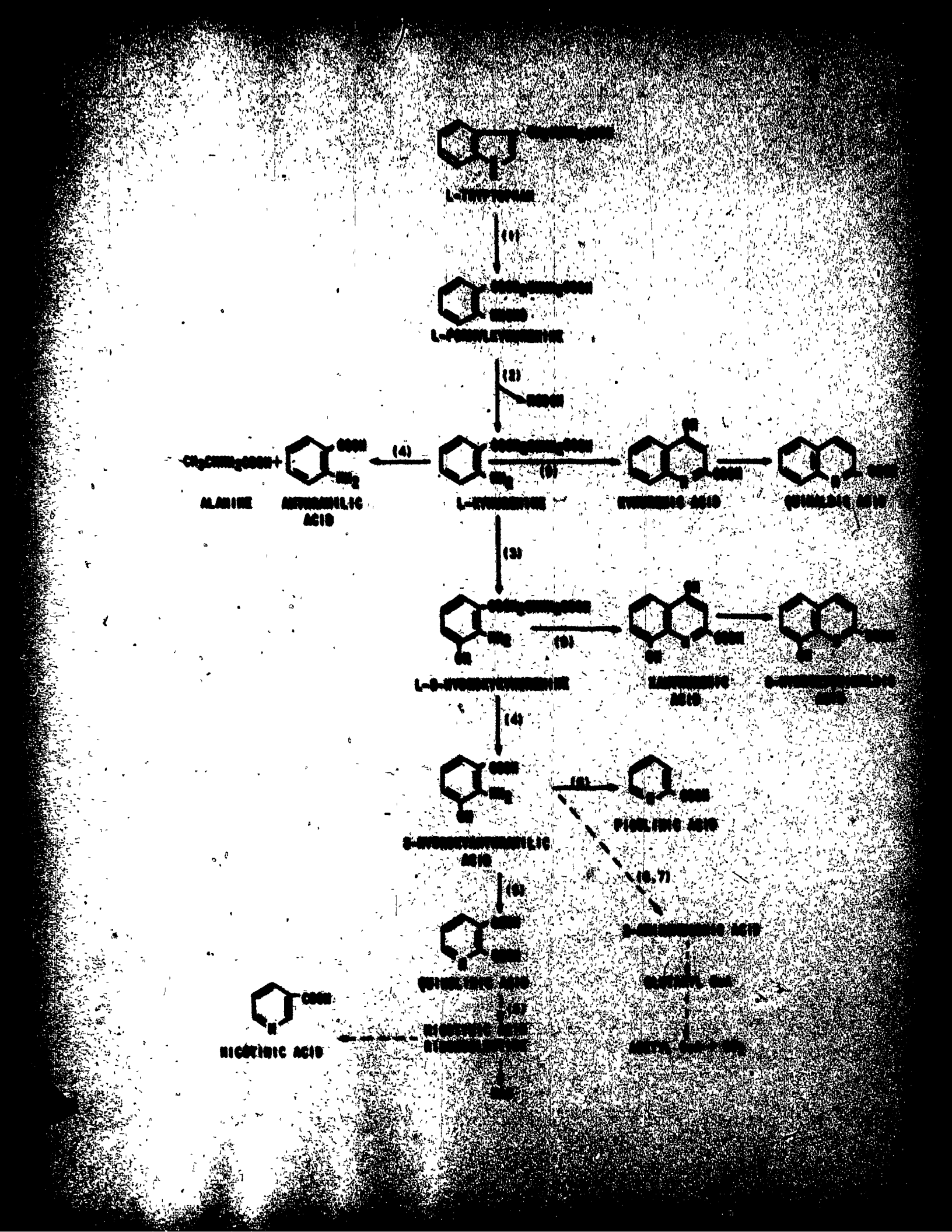
(D) protein synthesis, the method through which tryptophan is incorporated into the cellular proteins required for growth and development.

A. Catabolism of Tryptophan

Evidence for the degradation of tryptophan came from the laboratory of Kotake in Osaka (Ellinger and Matsuoka, 1920;

Figure 1

Catabolism of L-Tryptophan Along the Pyrrolase Pathway.


(1)
(2)
(3)
(4)
(5)
(6)
(6,7)
(7)
(8)
(9)
ALANINE
NICOTINIC ACID

Enzymes for the Catabolism of L-Tryptophan Along the Pyrrolase Pathway.

(Figure 1)

(1) L-Tryptophan Oxygenase (EC 1.13.1.12)

(2) Formamidase (EC 3.5.1.9)

(3) L-Kynurenine-3-Hydroxylase (EC 1.14.1.2)

(4) Kynureninase (EC 3.7.1.3)

(5) 3-Hydroxyanthranilate Oxygenase (EC 1.13.1.6)

(6) Picolinic Acid Carboxylase

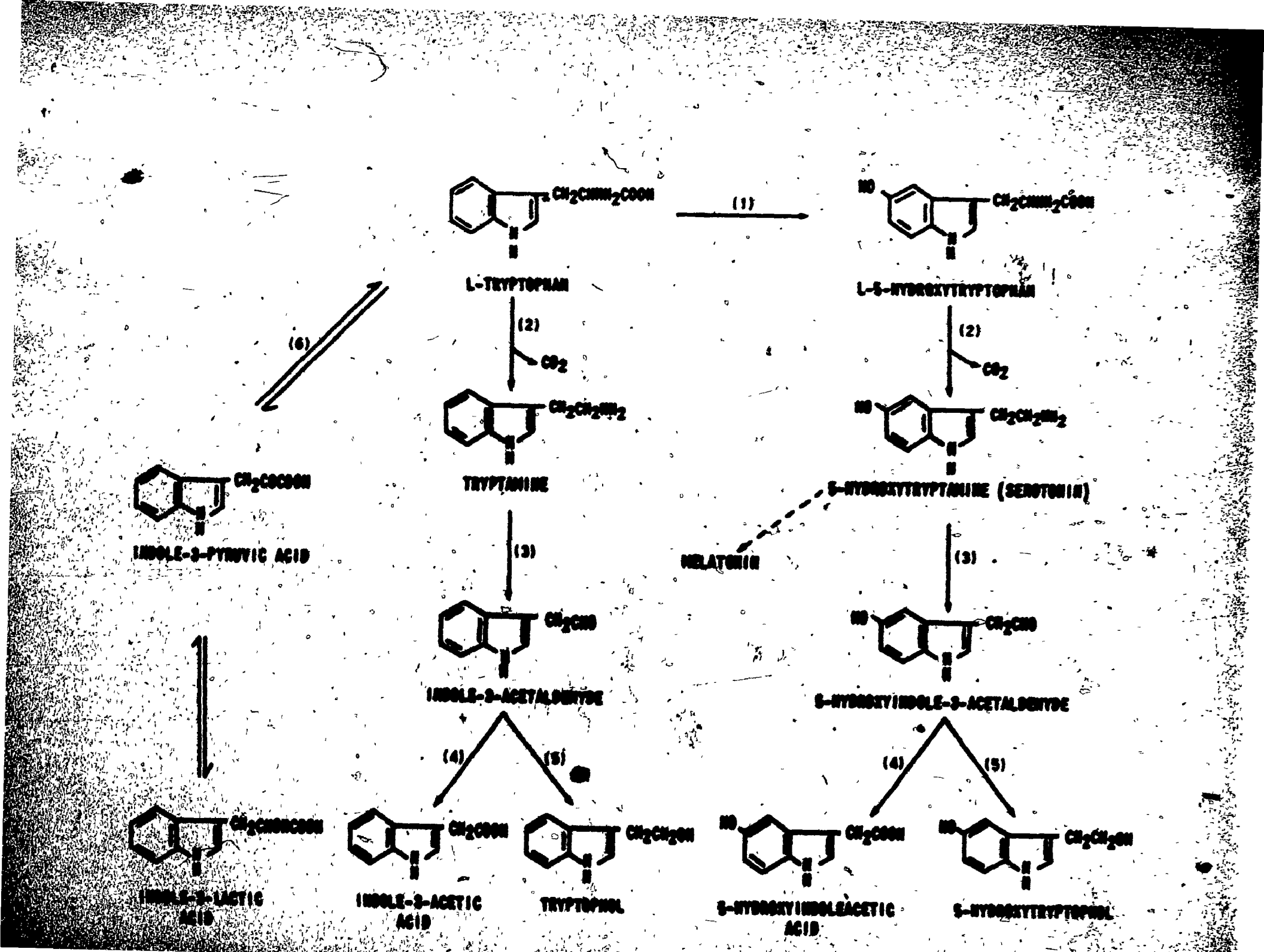
(7) 2-Hydroxymuconic-6-Semialdehyde Dehydrogenase

(8) Quinolinate Phosphoribosyltransferase

(9) Kynurenine Transaminase (EC 2.6.1.7)

Figure 2

Metabolism of L-Tryptophan Along Pathways in Which the Indole Ring Remains Intact.


L-TRYPTOPHAN
(1)
L-5-HYDROXYTRYPTOPHAN
(2)
CO_2
(6)
TRYPTAMINE
5-HYDROXYTRYPTAMINE (SEROTONIN)
INDOLE-3-PYRUVIC ACID
MELATONIN
(3)
INDOLE-3-ACETALDEHYDE
5-HYDROXYINDOLE-3-ACETALDEHYDE
(4)
(5)
INDOLE-3-LACTIC ACID
INDOLE-3-ACETIC ACID
TRYPTOPHOL
5-HYDROXYINDOLEACETIC ACID
5-HYDROXYTRYPTOPHOL

Enzymes for the Metabolism of L-Tryptophan Along Pathways in which the Indole Ring Remains Intact.

(Figure 2)

(1) Tryptophan-5-Hydroxylase (EC 1.14.3.3)

(2) Aromatic L-Amino Acid Decarboxylase (EC 4.1.1.28; EC 4.1.1.26)

(3) Monoamine Oxidase (EC 1.4.3.4)

(4) Aldehyde Dehydrogenase (EC 1.2.1.3)

(5) Alcohol Dehydrogenase (EC 1.1.1.1)
Aldehyde Reductase (EC 1.1.1.2)

(6) Transaminase

Matsuoka and Takemura, 1922). Using rabbits, this group showed that when tryptophan was administered subcutaneously, kynurenine and kynurenic acid could be isolated from the urine. From this work they proposed that the name tryptophan pyrrolase (EC 1.13.1.12) be given to the enzyme which converts tryptophan to kynurenine (Kotake and Masayama, 1936). It remained for Knox and Mehler (1950) to show that the formation of kynurenine from tryptophan is actually a two-step reaction. The first reaction, cleavage of the indole ring, is catalyzed by tryptophan pyrrolase or, as it is now officially named, tryptophan oxygenase. The second reaction, hydrolysis of the newly formed N-formylkynurenine to kynurenine, is catalyzed by the enzyme formamidase or aryl-formylamine aminohydrolase (EC 3.5.1.9).

(i) L-Tryptophan-2,3-Dioxygenase (EC 1.13.1.12)

Since the initial discovery of tryptophan pyrrolase, numerous studies have been devoted to characterization of this enzyme. It has been shown that pyrrolase is an iron porphyrin protein (Tanaka and Knox, 1959) and that the enzyme promotes the incorporation of molecular oxygen rather than hydrogen peroxide into the reaction products (Hayaishi, Rothberg, Mehler and Saito, 1957). Tryptophan pyrrolase is inducible; enzyme levels can be raised dramatically upon administration of tryptophan

(Civen and Knox, 1959), α-methyl-DL-tryptophan (Sourkes and Townsend, 1955), cortisone (Knox and Auerbach, 1955) or hydrocortisone (Civen et al., 1959). Recently Cho-Chung and Pitot (1967) have reported that pyrrolase is subject to feedback control by nicotinyl derivatives, the most potent of which is NADPH- a catabolic product of tryptophan.

Most of the studies detailed above have dealt with the liver enzyme. However, pyrrolase activity has been found in the intestine (Yamamoto and Hayaishi, 1967), in the eye (Ciusa and Barbiroli, 1966), in the kidney (Aslanyan, 1966) and, recently in brain. To date, it appears that although the pyrrolase pathway is the major catabolic route for tryptophan, it does not contribute significantly to tryptophan metabolism in brain.

(ii) Formamidase (EC 3.5.1.9)

N-formyl-L-kynurenine + H_2O → L-kynurenine + formic acid

Formamidase, the second enzyme in the catabolic pathway, has been detected in rat liver by Mehler and Knox (1950) as well as in several microorganisms (Hayaishi and Stanier, 1951; Jakoby, 1954). Using partially purified preparations of this enzyme and a variety of formyl compounds, Mehler et al. (1950) concluded that formylkynurenine is the natural substrate of this enzyme; a fact that was confirmed by Jakoby (1954) several years later.

(iii) L-kynurenine-3-hydroxylase (EC 1.14.1.2)

L-kynurenine + NADPH + H^+ + O_2 → L-3-hydroxykynurenine + NADPH + H_2O

Evidence linking kynurenine and 3-hydroxyanthranilic acid to niacin formation and thus to tryptophan catabolism was first provided by studies of Neurospora crassa mutants. Yanofsky and Bonner (1950) showed that 3-hydroxyanthranilic acid could produce niacin in a Neurospora crassa mutant which had a genetic block after kynurenine formation. Using these results, this group postulated that 3-hydroxykynurenine was the intermediate step between kynurenine and 3-hydroxyanthranilic acid (Bonner and Yanofsky, 1951). De Castro, Price and Brown (1956), using rat and cat liver mitochondrial preparations, were later able to demonstrate that 3-hydroxykynurenine could be formed from kynurenine and that this formation required NADPH. However, it was the work of Saito, Hayaishi and Rothberg (1957) with solublized preparations of this enzyme obtained from rat liver mitochondria which finally produced the overall reaction scheme outlined above. Stevens and Henderson (1959) have reported that the activity of this enzyme decreases significantly when rats are made riboflavin-deficient. Addition of riboflavin phosphate, FAD or boiled liver extract to an in vitro preparation of the enzyme fails to restore activity and hence the necessity of riboflavin for enzyme activity is still open to question.

(iv) Kynureninase (EC 3.7.1.3)

L-kynurenine
or
L-3 hydroxykynurenine
+ H_2O →
anthranilic acid
or
3-hydroxyanthranilic acid
+ alanine

Mammalian liver (Wiss and Fuchs, 1950; Wiss and Weber, 1956) and Neurospora crassa (Jakoby and Bonner, 1953) possess an enzyme which is capable of removing alanine from kynurenine and 3-hydroxykynurenine to yield anthranilic acid and 3-hydroxyanthranilic acid, respectively. This enzyme, now known as kynureninase, has been observed by Braunshtein, Goryachenkova and Paskhina (1949) to require pyridoxal phosphate. Similar to other pyridoxal phosphate requiring enzymes, it appears to act via a Schiff base intermediate (Longenecker and Snell, 1955).

(v) 3-Hydroxyanthranilate oxygenase (EC 1.13.1.6)

3-hydroxyanthranilic acid + O_2 →
{2-amino-3-carboxymuconic-6-semialdehyde} →
quinolinic acid

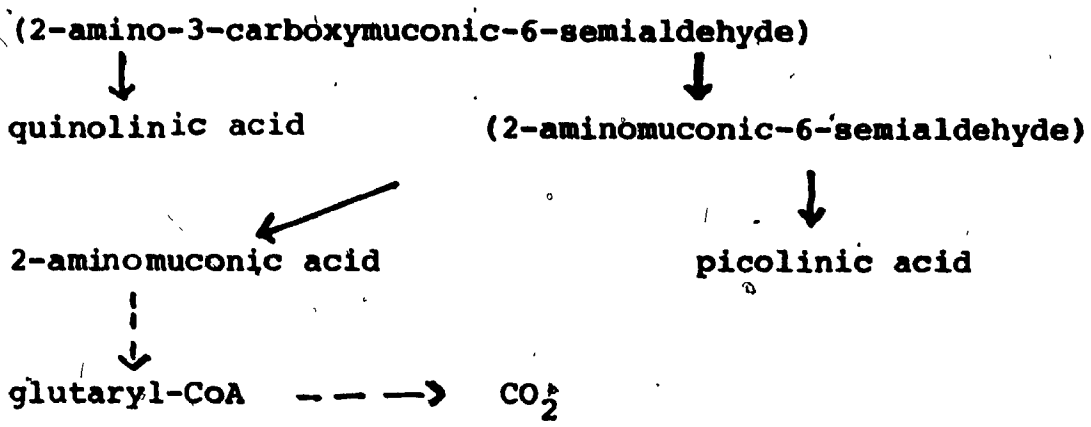
Marked increases in the urinary excretion of quinolinic acid following tryptophan administration to rats was first reported by Henderson (1949) and Henderson and Hirsch (1949). Shortly afterwards several investigators noticed that metabolism of 3-hydroxyanthranilic acid by rat liver preparations required

molecular oxygen and ferrous ions. Under these conditions the enzyme caused accumulation of a compound which absorbed maximally at 360 mμ (Miyake, Bokman and Schweigert, 1954; Mehler, 1956). Wiss and Bettendorf's (1957) isolation of the 2,4-dinitrophenylhydrazone derivative of this intermediate led Mehler (1956) to propose the now accepted mechanism of reaction. Mehler suggested that the oxidase cleaves the 3,4 double bond of the benzene ring and spontaneous cyclization of the open compound causes formation of the stable pyridine ring compound, quinolinic acid.

Hydroxyanthranilate oxygenase has now been purified from calf liver, (Vescia and Di Prisco, 1962) beef liver, (Stevens and Henderson, 1959a; Decker, Kang, Leach and Henderson, 1961).and beef kidney (Ogasawara, Gander and Henderson, 1966). These studies have shown that reducing agents such as glutathione, ascorbic acid or ferrous ions are required to maintain maximal activity during purification. It appears that the enzyme contains tightly bound ferrous iron which is necessary to catalyze the reaction but this ferrous iron is readily oxidized during purification procedures performed in the absence of reducing agent (Mitchell, Kang and Henderson, 1963).

(vi) Picolinic acid carboxylase

(vii) 2-hydroxymuconic-6-semialdehyde dehydrogenase



As detailed above, 3-hydroxyanthranilate oxygenase converts 3-hydroxyanthranilic acid to the open chain intermediate compound, 2-amino-3-carboxymuconic-6-semialdehyde. This intermediate has two fates: either it can cyclize spontaneously to form quinolinic acid or it can react enzymatically with picolinic acid carboxylase to form another unstable intermediate, 2-aminomuconic-6-semialdehyde. Similar to the oxygenase intermediate, this intermediate, 2-aminomuconic-6-semialdehyde, has two fates: either it can cyclize spontaneously to form picolinic acid or it can react with 2-hydroxymuconic-6-semialdehyde dehydrogenase to yield 2-aminomuconic acid. Via a series of additional reactions similar to those which occur during lysine metabolism, 2-aminomuconic acid can then be metabolized to glutaryl CoA, to acetyl CoA and ultimately to carbon dioxide.

Picolinic acid carboxylase activity was first detected by Mehler (1956) in preparations of guinea pig liver. Initially, he was able to distinguish only between the spontaneous formation of quinolinic acid and the enzymatic formation of picolinic acid. However, in a subsequent report, Mehler and May (1956), outlined the reaction sequences which led to the production of these two acids and proposed a structure for the active intermediate. It remained for Gholson, Nishizuka, Ichiyama, Kawai, Nakamura and Hayaishi (1962) to link this reaction sequence to the formation of glutaryl-CoA and carbon dioxide production. The second enzyme, 2-hydroxymuconic-6-semialdehyde dehydrogenase, an NAD-requiring enzyme was later purified and characterized by Ichiyama, Nakamura, Kawai, Honjo, Nishizuka, Hayaishi and Senoh (1965). Thus, it has been shown that mammalian species possess a mechanism for the degradation of tryptophan.

(viii) Quinolinate phosphoribosyltransferase

quinolinic acid → nicotinic acid ribonucleotide

The existence of this enzyme as demonstrated by Nishizuka and Hayaishi (1963a and 1963b) provided the missing link between quinolinic acid and niacin formation. It had been known for a long time that tryptophan could replace niacin in mammalian growth and development (Krehl, Teply, Sarma and

Elvehjem, 1945) but the mechanism by which these two compounds were related remained unknown for almost twenty years. This enzyme has been purified from liver (Nakamura, Ikeda, Tsuji, Nishizuka and Hayaishi, 1963; Gholson, Ueda, Ogasawara and Henderson, 1964) as well as from Pseudomonas (Packman and Jakoby, 1965). It has been shown that the enzyme interacts with quinolinic acid and 5-phosphoribosyl-1-pyrophosphate to form an intermediate compound, quinolinic acid ribonucleotide, which in turn is decarboxylated to nicotinic acid ribonucleotide. Nicotinic acid ribonucleotide can then be hydrolysed to nicotinic acid or it can react with ATP and glutamine to form diphosphopyridine nucleotide (NAD).

(ix) Kynurenine transaminase (EC 2.6.1.7)

L-kynurenine → kynurenic acid → quinaldic acid

L-3-hydroxykynurenine → xanthurenic acid → 8-hydroxyquinaldic acid

Kynurenic, xanthurenic, quinaldic and 8-hydroxyquinaldic acids have all been identified in the urine of humans, dogs, cats and rats (Brown and Price, 1956). Transamination of kynurenine and 3-hydroxykynurenine to kynurenic acid and xanthurenic acid occurs in rat liver mitochondria (Wiss, 1953), rat kidney (Mason, 1954), Neurospora crassa (Jakoby and Bonner, 1956) and Pseudomonas (Miller, Tsuchida and Adelberg, 1953). α-Ketoglutarate

appears to be the preferred amino group acceptor and the enzyme requires pyridoxal phosphate as coenzyme (Ogasawara, Hagino and Kotake, 1962; Ueno, Hayaishi and Shukuya, 1963). The presence of large amounts of quinaldic acid in human and rat urine following ingestion of kynurenic acid led Takahashi, Kaihara and Price (1956) and Kaihara and Price (1962), to postulate that kynurenic acid is dehydroxylated easily to quinaldic acid and that a similar reaction produces 8-hydroxyquinaldic acid from xanthurenic acid.

(x) 5-Hydroxytryptophan pyrrolase

During the past year several short communications have indicated that a tryptophan degradative pathway might be operative in brain. Tsuda, Noguchi and Kido (1972) have been able to demonstrate that 5-hydroxytryptophan can be converted to 5-hydroxykynurenine, 6-hydroxykynurenic acid and 4,6-dihydroxyquinoline by a supernatant fraction of rat brain. This conversion requires FAD and molecular oxygen but is somewhat inhibited by ascorbic acid.

From these results these investigators postulate that there is an enzyme system which can catalyze the oxygenative cleavage of 5-hydroxytryptophan to 5-hydroxykynurenine via 5-hydroxyformylkynurenine. Another group, Hirata, Tokuyama, Senoh and Hayaishi (1973) have reported on a tryptophan

2,3-dioxygenase which they have partially purified from rabbit ileum, stomach, lung and brain and which they have identified as distinct from the hepatic enzyme discussed previously. They have found that the enzyme requires methylene blue and ascorbic acid for maximal activity and that it is active on both D- and L-tryptophan, 5-hydroxytryptophan, tryptamine and serotonin. Thus it appears that a catabolic pathway for tryptophan may indeed be present in brain but identification of the metabolites of this pathway and determination of their physiological function await further elucidation. (See Addendum 1).

B. The 5-Hydroxyindole Pathway

The existence of the hydroxyindole pathway of tryptophan metabolism has been known for the last thirty-five years. Since it was first detected by Werle and Mennicken (1937) that tryptophan could be converted to a pressor amine compound, clinical interest in this pathway has produced a vast literature on the subject. Rapport, Green and Page (1948), isolated from beef serum a compound with vasoconstrictor properties. They identified this compound as serotonin. A few years later Erspamer and Asero (1952) showed that enterochromaffin cells of the intestinal mucosa produce a potent vascular hormone which they named enteramine but which they found later to be the same as serotonin. It was not long before Udenfriend, Clark and Titus (1953) were able to show that guinea pig kidney and tropical toads are able to hydroxylate tryptophan to 5-hydroxytryptophan. In a succeeding report Udenfriend, Titus, Weissbach and Peterson (1956) proposed that 5-hydroxytryptophan can be decarboxylated to serotonin and this can then be oxidized to 5-hydroxyindole-acetic acid. Using radioactive tryptophan they confirmed this pathway in dogs. A brief review of the enzymes of this metabolic route is presented here.

(i) Tryptophan-5-hydroxylase (EC 1.14.3.3)

L-tryptophan → L-5-hydroxytryptophan

Conversion of tryptophan to 5-hydroxytryptophan has been detected in liver (Freedland, Wadzinski and Waisman, 1961) in intestine and kidney (Cooper and Melcer, 1961), in brain (Gal, Morgan, Chatterjee and Marshall, 1964; Grahame-Smith, 1964a; Consolo, Garattini, Ghielmetti, Morselli and Valzelli, 1965; Weber and Horita, 1965), in human carcinoid tumors (Grahame-Smith, 1964b), in malignant mouse tumor cells (Lovenberg, Levine and Sjoerdsma, 1965) and in the pineal gland (Lovenberg, Jequier and Sjoerdsma, 1967).

In the liver, Freedland et al. (1961), Renson, Weissbach and Udenfriend (1962) and Freedland (1963) have all reported that tryptophan is hydroxylated by phenylalanine hydroxylase. The reaction requires reduced pteridine co-factor and NADPH. Phenylalanine is definitely the preferred substrate; the maximal rate of hydroxylation of phenylalanine is 30 times higher than for tryptophan. Mouse mast cells, however, have two hydroxylases: one for phenylalanine and one for tryptophan. Hosoda and Glick (1966) have purified the mast cell tryptophan hydroxylase and have shown that this enzyme requires tetrahydropteridine and molecular oxygen for maximal activity. Sato, Jequier, Lovenberg and Sjoerdsma (1967) have further reported that the enzyme requires ferrous ions. The intestinal enzyme from rat and guinea pig, characterized by Cooper et al. (1961), is very different. It requires cuprous ions and ascorbic acid

for activity and appears to function anaerobically.

Early attempts by Renson et al. (1962) to detect tryptophan hydroxylase activity in brain failed and led to the hypothesis that brain serotonin originated in other organs. It was Gal, Poczik and Marshall (1963) who first presented evidence for the in vivo hydroxylation of tryptophan by brain. Initial efforts to purify the enzyme failed to increase the enzyme activity significantly and resulted in poor enzyme yields. Nevertheless, Nakamura, Ichiyama and Hayaishi (1965), Gal, Armstrong and Ginsberg (1966) and Robinson, Lovenberg and Sjoerdsma (1968) were later able to show that the enzyme requires NADPH and tetrahydropteridine for activity and that it cannot hydroxylate phenylalanine. Jequier et al. (1969) reported that the Michaelis constant for tryptophan was 3×10^{-4} M. This led to the widely held idea that this enzyme is unsaturated under physiological conditions. Recently, Friedman, Kappelman and Kaufman (1972), using the naturally occurring co-factor have shown that the apparent Michaelis constant for the enzyme is 50 µM; a value in closer relation to the estimated concentrations of brain tryptophan. However, the enzyme is still believed to be only partially saturated in normal physiological conditions.

(ii) Aromatic L-amino acid decarboxylase (EC 4.1.1.28; EC 4.1.1.26)

L-5-hydroxytryptophan → 5-hydroxytryptamine (serotonin) + CO_2

Clark, Weissbach and Udenfriend (1954) demonstrated that a decarboxylase capable of forming serotonin from 5-hydroxytryptophan was present in guinea pig kidney, stomach, liver, intestine and lung as well as in rat, hog, rabbit and dog kidneys. They also reported that the enzyme requires pyridoxal phosphate for activity. Further evidence for this co-factor requirement was obtained by Buxton and Sinclair (1956); Buzard and Nytch (1957) and Weissbach, Bogdanski, Redfield and Udenfriend (1957) using vitamin B_6-deficient rats and chicks. These investigators showed that kidney decarboxylase activity in these animals is sharply decreased.

The initial purification of decarboxylase by Clark et al. (1954) indicated that there were two enzymes: one for 5-hydroxytryptophan and one for 3,4-dihydroxyphenylalanine. However, later studies by Yuwiler, Geller and Eiduson (1959); Werle and Aures (1959) and Rosengren (1960) suggested that 5-hydroxytryptophan and 3,4-dihydroxyphenylalanine were acted upon by the same enzyme. These reports led Lovenberg, Weissbach and Udenfriend (1962) to re-examine their earlier findings in guinea pig kidney and dog brain stem. They subsequently concluded that there is one enzyme in mammalian tissues which catalyzes the decarboxylation of 5-hydroxytryptophan, 3,4-dihydroxyphenylalanine, phenylalanine, tyrosine, tryptophan and

histidine and suggested that this enzyme be named "aromatic L-amino acid decarboxylase".

More recently, Christenson, Dairman and Udenfriend (1970; 1971) and Lancaster and Sourkes (1972) have purified this enzyme to electrophoretic homogeneity and have shown that it is a single protein with specificity such that it will act on both 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan as well as on phenylalanine and tryptophan. Christenson et al. (1971) have also prepared anti-serum to this enzyme and have shown that it is widely distributed in mammalian tissues. Bender and Coulson (1972) while acknowledging that the work of Christenson et al. (1970; 1971) provides positive proof for one enzyme protein, have suggested that the enzyme actually has a narrower range of specificity (just to 3,4-dihydroxyphenylalalanine and 5-hydroxytryptophan).

However, during the course of these investigations, a series of conflicting reports indicating the possibility of the existence of a multiplicity of decarboxylase enzymes have appeared. Awapara, Sandman and Hanly (1962) showed that 3,4-dihydroxyphenylalanine decarboxylases from liver and kidney are different with respect to substrate specificity, pH and co-factor requirements. Subcellular localization of this enzyme and of 5-hydroxytryptophan decarboxylase in various tissues is also different. Thus, kidney (Lovenberg et al., 1962), adrenal medulla (Blaschko, Hagen and Welch, 1955; Laduron and Belpaire, 1968) and brain (McGeer,

Bagchi and McGeer, 1965; Sims, Davis and Bloom, 1973) 3,4-dihydroxyphenylalanine decarboxylase are cytoplasmic. Kidney 5-hydroxytryptophan decarboxylase is also cytoplasmic (Lovenberg et al., 1962) but the brain enzyme is particulate according to Rodriguez De Lores Arnaiz and De Robertis (1964) and both soluble and particular according to Sims et al. (1973). These results indicate that the controversy as to whether there are one or two decarboxylase enzymes still remains to be resolved.

In most tissues, serotonin is further metabolized by the enzyme monoamine oxidase to 5-hydroxyindole acetaldehyde; this aldehyde is then either oxidized or reduced to the corresponding acid or alcohol. In the pineal, however, serotonin is the precursor of melatonin, the pineal hormone which has an antagonistic action toward melanocyte-stimulating hormone and which causes lightening of amphibian skin.

In the pineal, serotonin is N-acetylated and O-methylated by two enzymes (the former requires acetyl CoA as the acetyl donor and the latter requires S-adenosylmethionine as the methyl donor) to melatonin (McIssac and Page, 1959; Lerner, Case and Takahashi, 1960; Weissbach, Redfield and Axelrod, 1960; Axelrod and Weissbach, 1961).

(iii) Monoamine oxidase (EC 1.4.3.4)

5-hydroxytryptamine (serotonin) + O_2 + H_2O → 5-hydroxyindoleacetylaldehyde + NH_3 + H_2O_2

Hare (1928) was the first to describe an enzyme in mammalian livers which could catalyze the oxidative deamination of tyramine. A few years later, Blaschko, Richter and Schlossmann (1937a) described an adrenaline oxidase in liver, kidney and intestine and Pugh and Quastel (1937a) reported on the presence in brain of an enzyme that oxidized aliphatic amines. These investigators (Blaschko, Richter and Schlossmann, 1937b; Pugh and Quastel, 1937b; Kohn, 1937) ultimately concluded that the three enzyme systems are identical and that a single enzyme attacks adrenalin, tyramine and several aliphatic amines.

Since these initial studies, monoamine oxidase has been shown to occur in a wide variety of tissues. Liver, kidney, intestine, brain and blood platelets all have monoamine oxidase activity but other organs such as pancreas, heart, spleen and thyroid are active to some extent (Pugh et al., 1937a; Bhagvat, Blaschko and Richter, 1939; Latt, Rippey and Stacy, 1968). However, relative activities of different organs in different species vary widely.

This enzyme is present in liver mitochondria (Blaschko, 1952; Hawkins, 1952) and also as a soluble plasma protein (Tabor,

Tabor and Rosenthal, 1954; Weissbach, Redfield and Udenfriend, 1957). Many of the particulate amine oxidases have a flavin prosthetic group (Erwin and Hellerman, 1967; Tipton, 1968; Harada and Nagatsu, 1969; Kearney, Salach, Walker, Seng and Singer, 1971; Youdim and Sourkes, 1972) while the soluble plasma enzyme requires copper and pyridoxal phosphate as its prosthetic groups (Yamada and Yasunobu, 1962; Yamada and Yasunobu, 1963; Blaschko and Buffoni, 1965).

(iv) Aldehyde dehydrogenase (EC 1.2.1.3)

(v) Alcohol dehydrogenase (EC 1.1.1.1) and Aldehyde Reductase (EC 1.1.1.2)

5-hydroxyindoleacetaldehyde + NAD + H_2O → 5-hydroxyindoleacetic acid + NADH

5-hydroxyindoleacetaldehyde + NADH or NADPH → 5-hydroxytryptophol + NAD or NADP

5-hydroxyindoleacetaldehyde, formed by monoamine oxidase action, has two fates: either it can be oxidized to the corresponding acid (Weissbach et al., 1957; Eccleston, Moir, Reading and Ritchie, 1966; Feldstein and Williamson, 1968) or reduced to its alcohol (Kveder, Iskric and Keglevic, 1962; Eccleston et al., 1966; Feldstein et al, 1968; Duncan and Sourkes, 1974). The enzymes responsible for these metabolic

transformations are aldehyde dehydrogenase, alcohol dehydrogenase and aldehyde reductase.

Aldehyde dehydrogenase has been studied in greatest detail. Deitrich (1966) has found that it is an NAD-dependent enzyme. To date, it has been purified from bovine and monkey brain mitochondria (Erwin and Deitrich, 1966) and from pig brain (Duncan and Tipton, 1971). A number of aliphatic aldehydes, ranging from formaldehyde to palmitic aldehyde, as well as the aldehydes derived from serotonin, epinephrine and dopamine are substrates for this enzyme (Erwin et al., 1966).

Enzymatic reduction of aldehydes in liver occurs via an alcohol dehydrogenase which is NADH-dependent and inhibited by pyrazole (Sund and Theorell, 1963; Dalziel and Dickinson, 1965). Brain alcohol dehydrogenase similar to the liver enzyme has been detected (Raskin and Sokoloff, 1968). It too is NADH-dependent and pyrazole-sensitive. However, the activity of the brain enzyme is very low (Raskin and Sokoloff, 1972).

Eceleston et al. (1966) and Feldstein et al. (1968) have noticed that reduction of the aldehydes derived from certain brain amines requires NADPH rather than NADH. Recently Tabakoff and Erwin (1970) have reported on the presence of an NADPH-dependent, aldehyde-reducing enzyme in bovine brain tissue which is capable of converting aromatic and aliphatic aldehydes to their corresponding alcohol derivatives. Using partially purified preparations isolated from

rat brain, Tabakoff, Anderson and Alivisatos (1973) have just confirmed that the enzyme is indeed NADPH-dependent and pyrazole-insensitive, but that it is sensitive to pentobarbital. They have concluded that it is an aldehyde reductase. Thus, brain tissue appears to possess two distinctly different enzymes for aldehyde reduction: alcohol dehydrogenase and aldehyde reductase.

C. D-Tryptophan Metabolism

As early as 1931 Berg and Potgieter (1931) reported that rats receiving the DL form of tryptophan showed no significant difference in growth patterns from rats receiving only the L-isomer. Later, Schayer (1950) synthesized DL-tryptophan containing ^{15}N in the indole ring and separated this into the D and L isomers. He then showed that D-tryptophan is readily converted to L-tryptophan in the rat. Oates and Sjoerdsma (1961) carried this work further when they noticed that infusion of D-5-hydroxytryptophan into humans resulted in a significant increase in serotonin and 5-hydroxyindoleacetic acid levels. They postulated D-amino acid oxidase might convert D-5-hydroxytryptophan to 5-hydroxyindolepyruvic acid, which in turn could be transaminated to L-5-hydroxytryptophan. The newly formed L-5-hydroxytryptophan could then be decarboxylated by the enzyme, which is absolutely specific for the L-isomer, to yield serotonin. Two recent publications have provided additional support for this pathway

(Arendt, Contractor and Sandler, 1967; Yuwiler, 1973).

(i) D-amino acid oxidase (EC 1.4.3.3) and Transaminase

D-tryptophan + H_2O + O_2 → indolepyruvic acid + NH_3 + H_2O_2

or or

D-5-hydroxytryptophan 5-hydroxyindolepyruvic acid

indolepyruvic acid + glutamine → L-tryptophan + α-ketoglutarate

or or

5-hydroxyindolepyruvic L-5-hydroxytryptophan

While the above research was in progress, Lin, Pitt, Civen and Knox (1958) and Sandler, Spector, Ruthven and Davison (1960) reported that tryptophan-α-ketoglutarate transaminase and 5-hydroxytryptophan-α-ketoglutarate transaminase activities were present in rat liver preparations. A few years later Haavaldsen (1962) showed that rat brain extracts possess transaminase activity and Tangen, Fonnum and Haavaldsen (1965) were able to purify a transaminase from rat brain which was specific for tryptophan and 5-hydroxytryptophan.

In a similar fashion, evidence pointing to the existence of D-amino acid oxidase activity in various tissues began about 1935. Krebs (1935) was the first to report that fresh

liver and kidney slices were able to deaminate amino acids of the D series. Confirmation of kidney D-amino acid oxidase activity was published a few years later by Birkofer and Wetzel (1940). Rat brain oxidase activity was first detected by Edlbacher and Wiss (1944). This enzyme has since been found in human brain, kidney and liver by Dunn and Perkoff (1963).

Thus, the above studies have provided strong support for the metabolism of D-tryptophan by mammalian cells via the 5-hydroxyindole pathway once it has been converted to the L-isomer by the oxidase and transaminase enzymes.

(ii) D-tryptophan pyrrolase

D-tryptophan ---------- D-kynurenine

Considerable evidence has now accumulated in favor of the metabolism of D-tryptophan to D-kynurenine in intestine and liver. Early studies by Kotake and Ito (1937) showed that when rabbits were fed large doses of D-tryptophan, D-kynurenine could be isolated from the urine. These investigators were able to obtain conversion of D-tryptophan to D-kynurenine in extracts of intestinal mucosa but not in liver extracts. Studies of the liver enzyme by Knox et al. (1950) and Tanaka et al. (1959) showed that the enzyme was absolutely specific for the L-isomer. However, using a more sensitive chromatographic procedure for the separation of D- and L-kynurenine, Loh and Berg (1971; 1972)

have recently shown that rat liver can metabolize D-tryptophan to D-kynurenine. They have also reported that D-tryptophan pyrrolase is very similar to L-tryptophan pyrrolase but they have not as yet determined whether there are one or two enzymes present in liver. Intestinal conversion of D-tryptophan to D-kynurenine has been studied by Higuchi and Hayaishi (1967). They have concluded: (1) that this conversion is not caused by intestinal microflora and (2) that a D-tryptophan pyrrolase is present in the ileum region of rabbit intestine. They have shown that some differences exist between the intestinal D- and L-pyrrolase enzymes but they have not been able to determine conclusively whether there are really two enzymes in the intestine.

D. Comments

The above review of tryptophan metabolism and of the enzymes involved indicate that tryptophan is catabolized to niacin and CO_2 via the tryptophan pyrrolase pathway in the liver and also in the intestine. Most importantly, it is not believed to be a major degradative route in brain. During the course of the research for this thesis, interest in this pathway was generated by the idea that perhaps metabolites of the degradative route circulating in the plasma might affect brain tryptophan transport.

In brain then, there are only two metabolic routes to be considered at the present time: the 5-hydroxyindole pathway and the oxidase-transaminase shunt. Of these, the production of serotonin has generated the most interest since this compound is believed to be a neurotransmitter and since it has also been implicated as an important factor in depressive illnesses. It should be noted, however, that very little tryptophan is actually metabolized by the brain. Sjoerdsma, Weissbach and Udenfriend (1956) have estimated that only 1% of the total dietary tryptophan is metabolized to serotonin. Since serotonin is produced throughout the body, only a small fraction of that 1% is actually produced by the brain. Excluding protein synthesis, the 5-hydroxyindole pathway is probably the major route of tryptophan metabolism in brain.

Although tryptophan-5-hydroxylase is said to be the rate-limiting step in serotonin formation (Friedman, et al., 1972), numerous reports have shown that alterations in brain tryptophan levels can affect serotonin turnover in brain (Grahame-Smith, 1971; Tagliamonte, Tagliamonte, Perez-Cruet, Stein and Gessa, 1971; Curzon, Joseph and Knott, 1972). Thus, it appeared to us that the entry of tryptophan into brain might be a mechanism for the control of intracellular tryptophan concentrations and, therefore, study of the particular characteristics of this entry might be of considerable interest and importance.

3. Brain and Plasma Tryptophan Concentrations

The cellular concentrations of free tryptophan in the brain depends on several factors: (A) the influx and efflux of this amino acid in brain, (B) the rate of tryptophan metabolism and (C) the rate of tryptophan incorporation into protein. Despite these factors, the free tryptophan concentration of adult brain remains fairly constant. Schurr, Thompson, Henderson, Williams and Elvejhem (1950); Sourkes et al. (1970); and Grahame-Smith (1971) have determined that rat brain tryptophan concentrations range from 0.01 - 0.02 mmoles/kg.

During development the concentrations of most amino acids in brain tend to decrease (Guroff, 1972). Several years ago, Guroff and Udenfriend (1964) showed that the brain:plasma ratio for tryptophan decreases significantly with age. More recently, Tyce, Flock and Owen (1964) have reported that the brain tryptophan concentration of fetal and newborn rats is about three times that of the adult concentration. Within the first three days of life they showed that there is a rapid decrease in tryptophan concentration followed by a steady increase which levels off at adult values in approximately three weeks; the adult concentrations however, are always considerably smaller than those of the newborn.

The total plasma tryptophan concentration is much higher than the brain concentration, ranging from 0.05-0.10 mmoles/l

McMeanamy and Oncley (1958) have reported that only 20% of the total plasma tryptophan is in a freely diffusible form. The ratio of free:bound serum tryptophan is sensitive to changes in temperature, pH and ionic strength (McMenamy et al., 1958), to various drugs such as probenecid, salicylate and aspirin (Tagliamonte, Biggio and Gessa 1971); as well as to non-esterified fatty acids (Lipsett, Madras, Wurtman and Munro, 1973; Curzon, Friedel and Knott, 1973).

Currently a number of investigators are postulating that brain tryptophan concentration is controlled by the level of free serum tryptophan. These groups base their hypothesis on the following observations: 24-hour food deprivation in rats (Knott and Curzon, 1972; Tagliamonte, Biggio, Vargiu and Gessa, 1972), hepatic devascularization in pigs (Curzon, Kantameni, Winch, Rojas-Bueno, Murray-Lyon and Williams, 1973), or 3-hour immobilization of rats (Knott et al., 1972) causes significant increases in free serum tryptophan and in brain tryptophan but the total plasma tryptophan concentration remains unchanged. This hypothesis, however, has been questioned by the study of Madras, Cohen, Fernstrom, Larin, Munro and Wurtman (1973). Madras et al. (1973) have shown that a high carbohydrate diet causes decreased free serum tryptophan but increased brain tryptophan concentrations in rats. They argue that when rats are fed such a diet, the non-esterified fatty acid concentration in the

serum is decreased. Since non-esterified fatty acids are known to alter the affinity of tryptophan for serum albumin (Curzon, Friedel and Knott, 1972) then decreases in these fatty acids cause increased binding of tryptophan and a lower free concentration. Thus, they conclude that free serum tryptophan does not predict changes in brain tryptophan caused by such physiological inputs as eating but that free serum tryptophan might be correlated with brain tryptophan after treatments such as drug administration or fasting.

4. Introduction to Transport

Studies indicating that the brain cell tryptophan concentration remains fairly constant are further proof for the role of the cell membrane in the maintenance of cellular homeostasis. For many years it has been evident that molecules and ions penetrate cell membranes at varying rates and with varying degrees of selectivity. This evidence has given support to the now accepted concept that cell membranes are not inert barriers separating intracellular and extracellular environments but rather that they are dynamic physiological structures capable of facilitating or obstructing the entry (or exit) of a molecule or ion.

A. Simple or Passive Diffusion

The simplest form of transport across a cell membrane

is "simple or passive diffusion" (Danielli, 1954). In such a case, the substance being transported moves along its electrochemical potential (or concentration) gradient until the gradient disappears. This process requires no energy expenditure by the cell and results in an equivalent concentration of the transported substance on either side of the membrane. Owing to the complexity of cell membranes, however, this type of transport occurs infrequently.

B. Facilitated Diffusion

Usually substances which diffuse across cell membranes do so by "facilitated diffusion" (Danielli, 1954). In facilitated diffusion, the net thermodynamic result is equivalent to the process of simple diffusion with the substance being transported moving in the direction determined by the electrochemical potential (or concentration) gradient. As in the case of simple diffusion, no input of cellular energy is required for transport. By facilitated diffusion, a 1:1 ratio of the intracellular:extracellular concentration of the transported substance is attained but the rate at which this equilibrium is achieved is much greater than would occur by simple diffusion. For this reason investigators have postulated that cell membranes possess mechanisms whereby a substance can be "carried" into a cell. The exact nature of these carriers is still the subject of much study but

one can say that at the very least, they enhance or facilitate the transport of a substance across the cell membrane. In the case of facilitated diffusion, these carriers may distinguish between stereoisomers, allow structurally analogous compounds to reduce the movement of a substance across the cell membrane, and can be affected by certain metabolic inhibitors. Also, in a facilitated diffusion process, the rate of penetration by a transported substance may not be directly proportional to the concentration of that substance in the extracellular fluid but rather may reach a limiting value. In a simple diffusion process no saturation value can occur.

C. Active Transport

A number of substances, notably ions, sugars and amino acids, are concentrated inside various cells; that is, they are transported through cell membranes to higher electrochemical potentials (or concentrations). Such transport processes, known as "active transport" require the expenditure of cellular energy either from the hydrolysis of cellular ATP and/or from the potential energy of an ion gradient. These processes are characterized by saturation kinetics which can be described by adaptations of the Michaelis-Menten equation. Analogs of the substance being transported usually inhibit competitively.

Current hypotheses concerning the mechanism by which a substance is actively transported into a cell all support the idea of a carrier within the cell membrane. To date two different concepts of this carrier have been proposed: either it is a mobile carrier which moves through the membrane or it is fixed in the membrane and the substance gets into the cell by moving along adsorptive sites on the carrier. Most researchers today favor some variation of a mobile carrier concept and it is this concept which will be discussed further here.

D. Mobile Carrier Hypotheses

Mobile carrier hypotheses postulate that the substance to be transported binds to a receptor site on the carrier which is situated on the outer side of the cell membrane. This carrier complex then translocates through the membrane. Inside the cell the substance is released. In the case of a facilitated diffusion process, the movement is down a concentration gradient, i.e., the carrier returns empty to the outer side of the membrane. In an "exchange diffusion" process (Ussing 1947; 1949), the carrier picks up another molecule of the same substance ("homoexchange") or a structurally analogous substance ("heteroexchange") at the inner side of the membrane and carries it to the outside where the substance is released. In an active transport process, the substance is carried into the cell against its concentration gradient.

E. Energy Requirements for Active Transport

The driving force for this concentration process has been the object of numerous exhaustive studies, none of which has answered the question conclusively. Most investigators agree that cellular energy must be expended. The energy source is believed to be ATP because metabolic inhibitors (cyanide, 2,4-dinitrophenol, arsenate, iodoacetate, etc.) of ATP formation inhibit the active transport process drastically. In addition to ATP, however, the intracellular and extracellular Na^+ and K^+ concentrations are important for maximal accumulation.

F. The Sodium-Gradient Hypothesis

A number of years ago, Crane (1965) postulated that a sugar or amino acid could be concentrated by an intestinal cell in the following manner: a carrier with two binding sites is present on the outer side of the cell membrane. One site is specific for the sugar or amino acid and its analogs. The other site binds Na^+ or K^+ ions. In order for translocation to occur, both binding sites must be occupied. When this occurs, the substrate-ion-carrier complex moves through the membrane (in other words, the substrate and ion are "co-transported") and releases the substrate and ion into the cell. Now the ion binding site, however, has a different affinity for extracellular

Na^+ and K^+ . If Na^+ is bound to the carrier on the outside, the conformation of the complex is such that the carrier moves rapidly through the membrane and the substrate is accumulated within the cell. When K^+ is bound to the carrier, the conformation of the complex is such that translocation occurs slowly and very little substrate is accumulated. Crane further postulated that since the K^+ concentration inside the cell is 10-20 times greater than the Na^+ concentration, the $Na^+ - K^+$ ATPase present in the cell membrane pumps out the Na^+ with the concurrent hydrolysis of ATP and it is this energy expenditure which maintains the substrate concentrated inside the cell against its gradient.

Many investigators concur with this hypothesis, i.e., that it is the downhill Na^+ gradient which drives the uphill accumulation inside cells of sugars and amino acids. Thus, Vidaver (1964a; 1964b), Vidaver and Shepherd (1968) for glycine transport into pigeon erythrocytes; Eddy, Mulcahy and Thomson (1967) for glycine transport into Erhlich ascites cells and Curran, Schultz, Chez and Fuisz (1967) for alanine, valine and leucine transport into rabbit ileum provide results which agree in principle with this hypothesis. More recently however, Potashner and Johnstone (1971) have obtained data which conflict with this theory. They report that cellular ATP and extracellular Na^+, rather than a Na^+ gradient are required for the optimal accumulation of glycine and methionine by Ehrlich ascites cells.

These investigators suggest that their results indicate that both Na^+ and ATP act directly on the step involved in amino acid uptake into the cell.

5. Tryptophan Transport into Various Tissues

A. Intestine

Early evidence for the accumulation of tryptophan was reported by Spencer and Samily (1957). Using everted hamster intestinal sacs, they showed that at low concentrations (5 mM) tryptophan could be transported from the mucosal to the serosal side of the membrane and accumulated against its gradient. Similar to the uptake of phenylalanine and tyrosine, this transport process is completely inhibited by high tryptophan concentrations (20 mM); a phenomenon which had led Wiseman (1956) to report initially that tryptophan was the only amino acid not actively transported by the intestine. Spencer et al. (1957) also observed that tryptophan is transported best by middle sections of the intestine when compared with studies on sections from either end. On the basis of their results, they postulated that the four aromatic amino acids (tryptophan, tyrosine, phenylalanine and histidine) share at least one common step in their

intestinal absorption. Spencer, Bow and Markulis (1962) and Lin, Hagihira and Wilson (1962) later showed that tryptophan transport is structurally specific; the intestinal process requires the α-amino group and will not transport the D-isomer of tryptophan. Cohen and Huang (1964) observed that tyrosine and phenylalanine inhibit hamster intestinal transport of tryptophan competitively; that this transport process is located mainly on the mucosal side of the membrane and that replacement of Na^+ by Li^+ or K^+ markedly inhibits transport. Methylated and N-chloroacetylated derivatives of tryptophan can be transported by this preparation at a much slower rate than L-tryptophan but D-tryptophan, 5-hydroxy-D,L-tryptophan, tryptamine and 5-hydroxytryptamine are not transported at all. Using everted sacs of rat small intestine, Munck (1966) concluded that tryptophan is a substrate for the carrier of the diamino acids (arginine and lysine) as well as the neutral amino acids (methionine and leucine). More recently, Reiser and Christiansen (1973) have shown that epithelial cells of the rat intestine have two distinct alanine exchange transport systems, one for neutral amino acids and one for basic amino acids. Tryptophan, a large neutral amino acid has a weak ability to exchange with intracellular alanine; also 1 mM alanine inhibits the uptake of 1 mM tryptophan by approximately 20%.

B. Ehrlich ascites cells

Riggs, Coyne and Christensen (1954) were the first to elucidate tryptophan transport into Ehrlich ascites cells. They found: (1) that tryptophan is strongly accumulated by these cells; (2) that D,L-tryptophan is more weakly accumulated; (3) that the primary amino group is absolutely essential for tryptophan entry and (4) that loss of a carboxyl group or addition of a methyl group does not make the membrane impermeable to such a tryptophan derivative. Jacquez (1961) later reported that at low extracellular tryptophan concentrations (1 mM), the initial rate of uptake is linear for less than one minute. With high extracellular tryptophan concentrations (30 mM) the initial rate of uptake is linear for 10-15 minutes and steady state is reached only after 120 minutes of incubation. Low concentrations (1 mM) of histidine and leucine stimulate the rate of uptake of 1 mM extracellular tryptophan whereas high extracellular concentrations (5 mM) of these amino acids decrease tryptophan uptake by 13-15%. Oxender and Christensen (1963) have noted that tryptophan uptake is almost completely inhibited by phenylalanine whereas glycine and alanine caused less than 40% inhibition.

C. Escherichia coli

An inducible tryptophan transport system in Escherichia coli has been observed by Boezi and De Moss (1961).

This system has the following properties: (1) tryptophan accumulation is stimulated by Mg^{2+} inhibited by Mn^{2+} and unaffected by Ca^{2+} or Na^{+}; (2) 2,4-dinitrophenol, sodium azide, pyruvate and glucose inhibit tryptophan uptake whereas formate stimulates tryptophan accumulation. These investigators conclude that it is an energy requiring process since metabolic inhibitors decrease transport. They suggest that the glucose and pyruvate inhibition may actually be caused by a metabolic intermediate of these compounds which acts on the transport process; (3) phenylalanine and methionine stimulate tryptophan uptake whereas serine, valine, glycine, D-tryptophan, 5-methyl-D, L-tryptophan, 5-hydroxy-D,L-tryptophan and indole decrease uptake, and tryptamine and anthranilate have no effect. (4) the system is inducible because the tryptophan accumulating capacity increases with increasing concentrations of tryptophan in the growth medium. This induction is inhibited by chloramphenicol.

D. Human fibroblasts

Since Hartnup disease in humans is characterized by impaired renal and intestinal absorption of tryptophan, Groth and Rosenberg (1972) set out to discover whether this impairment might be reflected in the skin fibroblasts of patients suffering from this disease. They have been able to demonstrate that tryptophan uptake by cells from a child with Hartnup disease is

identical with that of cells from a normal subject. Furthermore, they reported that this uptake process is saturable and that there are two systems: a low affinity system with a K_m of 12.5 mM and a high affinity system with a K_m of 0.02 mM. These uptake processes are both Na^+ independent. Ouabain and 2,4-dinitrophenol do not inhibit either system; cyanide inhibited the high affinity system but not the low affinity system and p-chloromercuribenzoate affected both systems. Thus, they concluded that the gene loci mutant in this disease is not expressed in cultured human fibroblasts.

E. Brain

The early *in vivo* and *in vitro* studies of aromatic amino acid transport in rat brain were done by Chirigos, Greengard and Udenfriend (1960), Guroff, King and Udenfriend (1961) and Guroff and Udenfriend (1962). Initially these investigators noticed that *in vivo* accumulation of L-tyrosine is inhibited by L-tryptophan and to a lesser extent by D-tryptophan (Chirigos *et al.*, 1960). Additional studies of tyrosine uptake by rat brain slices confirmed this tryptophan inhibition (Guroff *et al.*, 1961). More direct investigations of tryptophan transport showed that *in vivo* administration of L-tryptophan causes marked increases in brain and plasma concentrations of this amino acid. When D-tryptophan is injected, however, much

less enters the brain (Guroff et al., 1962).

Until 1970 there were no further studies specifically of brain tryptophan transport. During the intervening years (1961-1968), however, thorough investigations directed by Abel Lajtha in New York produced a generalized concept of amino acid transport into brain. In the course of these studies, the effects of tryptophan on the brain transport of a number of amino acids was reported.

Thus, Blasberg and Lajtha (1965; 1966) and Neame (1966; 1968) have established that each amino acid does not have its own carrier or transport site on the brain cell membrane. Instead, it appears that there are at least six transport sites which can be readily distinguished and which transport groups of amino acids of similar structure and charge. These groups or classes are: (1) acidic (aspartic, glutamic); (2) small neutral (alanine, glycine, serine, threonine, α-aminoisobutyric, cycloleucine); (3) large neutral (leucine, methionine, tryptophan, tyrosine, phenylalanine); (4) small basic (2,3-diaminopropionic, 3,4-diaminobutyric); (5) large basic (ornithine, lysine, arginine) and (6) GABA. In addition, there may be a seventh group or a sub-class of the neutral hydroxyproline, sarcosine, betaine and histidine.

Blasberg et al. (1965; 1966) have further reported that these groups can be distinguished whether the steady state

accumulation or initial influxes of these amino acids into slices are studied. However, there appears to be some overlap of specificity. They have concluded, therefore, that each amino acid has a high affinity for a primary transport site and a much lower affinity for a secondary transport site. These investigators have also noted the similarity between their grouping of the amino acids and those of Oxender et al. (1963) and Christensen (1964) for Ehrlich ascites cells. Thus, they suggest that the "A" and "L" systems for neutral amino acid transport in Ehrlich ascites cells correspond to their small and large neutral groups respectively, while the "A+" and "L+" groups correspond to their small and large basic amino acid groups respectively. In vivo experiments by Battistin, Grynbaum and Lajtha (1971) have shown that the same type of substrate specificity operates in the living brain. Studies of the substrate specificity of exit of α-aminoisobutyric acid, leucine, lysine and glutamic acid by Levi, Blasberg and Lajtha (1966) have indicated that the pattern for exit from slices is similar in many ways but not identical to the pattern for initial influx and steady state accumulation. More recently Crnic, Hammerstad and Cutler (1973), using a number of other amino acids have reached the same conclusion.

Following the initial in vivo account of a concentrative mechanism of tryptophan uptake by rat brain (Guroff et al.,

1962), Neame (1964) reported that tryptophan inhibits histidine uptake into rat brain slices and Blasberg et al. (1965) showed that 10 mM medium tryptophan concentrations inhibits the uptake of 2 mM medium concentrations of the following amino acids: glycine, alanine, proline, leucine, phenylalanine, arginine and lysine also into mouse brain slices. Thus, at the outset of this thesis very little was known about tryptophan transport in brain.

However, several short communications and abstracts had indicated: (1) that 10-20 mM concentrations of glucose, pyruvate, lactate and oxaloacetate stimulate the concentrative uptake of tryptophan into brain slices while 20 mM α-ketoglutarate, succinate and fumarate have no effect, and N_2, sodium azide, sodium cyanide and 2,4-dinitrophenol significantly inhibit uptake (Barbosa, Joanny and Corriol, 1968); (2) that the K_m for tryptophan transport into brain slices increases when Na^+ is absent from the medium. Also, addition of 0.1 mM ouabain or removal of K^+ from the incubation medium decrease tryptophan uptake (Joanny, Barbosa and Corriol, 1968); (3) that tyrosine and phenylalanine inhibit tryptophan uptake while alanine, tyrosine and phenylalanine stimulate tryptophan efflux from slices (Joanny, Corriol and Hillman, 1969); and (4) that tryptophan can be actively transported into rat brain synaptosomes (Grahame-Smith, Parfitt, Warnock and Taylor, 1969). Full length papers detailing the preceding findings have been published recently

by the aforementioned investigators. These results will be discussed further in a later section of this thesis in conjunction with results obtained by this author.

EXPERIMENTAL

6. Materials

A. Radioactive Compounds

Inulin-{carboxyl-^{14}C} specific activity 2-4 mCi/g and L-tryptophan-{carboxyl-^{14}C} specific activity 9-12 mCi/mmole were both obtained from New England Nuclear Corporation, Boston, Massachusetts.

B. Other Chemicals

New England Nuclear Corporation, Boston, Massachusetts also supplied 1,4-bis-2-(4 methyl-5-phenyloxazole)-benzene (dimethyl POPOP) and 2,5 diphenyloxazole (PPO).

Glycine, L-histidine, L-tyrosine, L-phenylalanine, L-methionine, L-alanine, L-glutamine, L-lysine monohydrochloride, L-valine, L-arginine hydrochloride, L-isoleucine, L-threonine, L-glutamic acid, L-serine, L-proline, hydroxy-L-proline, DL-α-methyltryptophan monohydrate, kynurenic acid, xanthurenic acid, D,L-indole-3-lactic acid, nicotinic acid, picolinic acid, indole-3-acetic acid creatinine sulfate and inulin were purchased from Sigma Chemical Company, St. Louis, Missouri.

L-cysteine hydrochloride hydrate, γ-aminobutyric acid, L-kynurenine (as the free base), L-3-hydroxykynurenine, N' formyl-L-kynurenine monohydrate, anthranilic acid, tryptamine hydrochloride, serotonin creatinine sulfate complex, L-5-hydroxytryptophan, 5-hydroxyindoleacetic acid the cyclohexylammonium

salt with methanol, D-fructose, D-lactic acid (lithium salt), and pyruvic acid (sodium salt) were obtained from Calbiochem, Los Angeles, California.

The following items were bought from Nutritional Biochemicals Corporation (N.B.C.) Cleveland, Ohio: p-aminobenzoic acid, D-biotin, folic acid, thiamine hydrochloride, vitamin B_{12} 0.1% trituration in mannitol, D-calcium pantothenate, menadione (Vitamin K), quinolinic acid and casein hydrolysate.

Ouabain (Strophanthin G), DL-p-chlorophenylalanine, 4-methyl-D,L-tryptophan and 6-methyl-D,L-tryptophan were supplied by Mann Research Laboratories, New York City, New York.

Quinaldic acid, choline chloride, nicotinic acid, riboflavin and D,L-tryptophan were products of Eastman Organic Chemical Distillation Products Industries, Rochester, New York.

2,4-dinitrophenol, sodium cyanide, lithium chloride, naphthalene,dioxane and D,L-tyrosine were ordered from Fisher Scientific Co. Ltd., Montreal, Quebec.

α-methyl-5-hydroxy-D,L-tryptophan and L-leucine were obtained from Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

The Upjohn Company, Kalamazoo, Michigan provided 4-hydroxy-D,L-tryptophan and α-methylserotonin.

BDH Chemicals Ltd., Poole, England supplied cesium chloride, rubidium chloride, thallous chloride and iodoacetamide.

Succinate was obtained from Eastman Kodak Co., Rochester, New York while D,L-phenylalanine was purchased from J.T. Baker Chemical Co., Phillipsburg, New Jersey. Triton X-100 was ordered from American Hospital Supply Corp., Harleco Division, Philadelphia, Pennsylvania. Crystalline sodium citrate and inositol were bought from Anachemia Chemicals Ltd., Montreal, Quebec. Machery Nagel & Co., Germany was the supplier of Cellulose 60 (Cellulosepulver MN 300). Sucrose, Benson's corn starch, Crisco fat and Mazola corn oil were grocery products from Steinberg's Ltd., Montreal, Quebec. Pyridoxine hydrochloride was obtained from Chas. Pfizer and Co., New York, New York. L-tryptophan was a product of the Matheson Coleman and Bell Division of The Matheson Co. Inc., Norwood, (Cincinnati) Ohio.

All other chemicals were reagent-grade chemicals which were obtained locally and which were used without further purification.

C. Animals

Male albino rats of the Sprague-Dawley strain (130-150 g) were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec, at least one day before use. The rats were fed standard Purina rat chow ad libitum, except where noted.

Adrenalectomized or hypophysectomized animals (130-150 g) were obtained from the same company two days after the respective organs had been removed. The adrenalectomized rats were fed

standard Purina chow but were given saline to drink. The hypophysectomized animals were treated in the same manner as normal rats. Both groups of animals were kept for about five days before use. During this period body weights were recorded; hypophysectomized animals which showed significant weight gain were not included in the experiments.

7. METHODS

A. Tissue Preparation and Incubation

Rats were decapitated two at one time and the heads were taken immediately into the cold room (approximately 4°C). The brains were quickly removed and placed on Parafilm in covered Petri dishes containing moistened filter paper. Using a Stadie-Riggs microtome, two dorsal surface slices and two lateral surface slices, 0.5 mm thick, were obtained from each brain. One dorsal and one lateral slice were then weighed together (50-100 mg) and placed in a small flask with 3.0 ml of Krebs-Ringer phosphate medium (pH 7.0) cooled to 4°C and containing the following: NaCl, 145 mM; KCl, 5.5 mM; $CaCl_2$, 1.5 mM; $MgSO_4$, 1.4 mM; Na_2HPO_4, 10 mM; glucose, 10 mM. The time interval from sacrifice to immersion of the cortical slices in the incubation medium was routinely held to 10-12 min. The flasks were pre-incubated at 37°C on a Dubnoff shaking water-bath in an atmosphere of pure oxygen or nitrogen. After 10 min, the medium was removed and a fresh solution of Krebs-Ringer phosphate, containing 10 mM glucose, carrier tryptophan and L-tryptophan-{carboxyl-^{14}C}, was added; the specific activity of the tryptophan was 9-12 μCi/μmole.

Transport was halted by placing the flasks in ice at the end of the incubation. The medium was immediately withdrawn and stored for subsequent counting in Bray's (1960)

solution. The slices were removed, drained of excess fluid and weighed to determine the amount of swelling before being homogenized in 2 ml of an ethanol-trichloroacetic acid mixture containing (v/v) 1 part of 95% (v/v) ethanol to 4 parts of 5% (w/v) trichloroacetic acid. The homogenates were allowed to stand for 60 min before being centrifuged at 2,000 g for 10 min in an International Bench Centrifuge. The pellets were treated a second time as already described.

B. Determination of Tryptophan Uptake

Portions of the medium (0.1 ml) and of the two tissue extracts (0.5 ml) were added to 10 ml volumes of a modified Bray's (1960) solution {containing 333 ml of toluene, 333 ml of dioxane, 333 ml of 95% (v/v) ethanol, 80 g of naphthalene, 50 mg of 1,4-bis-2-(4 methyl-5-phenyloxazole)-benzene (dimethyl POPOP) and 5g of 2,5 diphenyloxazole (PPO)} or 10 ml volumes of a toluene-Triton X-100 solution {containing 700 ml toluene, 300 ml Triton X-100, and 5 g PPO} and were counted in a Beckman LS-250 Liquid Scintillation Counter. Efficiency was calculated to be 90 per cent using a toluene -^{14}C standard solution.

C. Estimation of the Inulin Space Correction Factor

Rat brain cerebral cortex slices were prepared as described. One dorsal and one lateral slice were weighed

together (50-100 mg) and were placed in a small flask containing 2.9 ml of the Krebs-Ringer phosphate glucose medium outlined above which had been cooled to 4°C. The flasks were transferred to a Dubnoff shaking waterbath at 37°C and a 0.1 ml aliquot of inulin -(carboxyl-^{14}C), 2-4 mCi/g was immediately added to each. In experiments where cold carrier inulin was added as well as the radioactive inulin, the 0.1 ml aliquot was of a 0.5% inulin solution containing inulin-(carboxyl-^{14}C), 2-4 mCi/g. Incubations were carried out in an atmosphere of either pure oxygen or nitrogen. Following the incubation period which ranged from 30 min to 180 min, the beakers were removed and placed on ice. The medium was withdrawn and the slices were treated in the same manner as for the transport experiments detailed above. The media and tissue extracts containing radioactive inulin were always allowed to stand overnight at 4°C before aliquots of these were added to either Bray's (1960) solution or toluene-Triton X-100 solution, and counted with greater than 90% efficiency. Since inulin precipitates quite readily from scintillation mixtures, Cohen (1972) advises that the media and tissue extracts stand overnight so that the inulin can hydrolyze to fructose.

D. Dry Weight Determination

Tissue slices were prepared and incubated as described above in the estimation of the inulin space correction factor.

Following incubation, the slices were blotted, placed on tared aluminum weighing dishes and were dried overnight at 105°C. The dry weight of cerebral cortex slices was 18.5% ± 0.8 (S.E.) (n=18) of the initial fresh weight of the tissue. The duration of incubation had no significant effect on the dry weight. Similarly, the dry weights of slices which were determined directly, i.e., without incubation, were also not significantly different.

E. Incorporation of L-Tryptophan-(Carboxyl-^{14}C) into Protein in Vitro

Rat brain cerebral cortex slices were prepared and incubated for 15 minutes as described. Following incubation, free soluble tryptophan was extracted from the slices by two successive treatments with trichloroacetic acid-ethanol solution as outlined above. Radioactivity remaining in the pellets after the second extraction was assumed to be tryptophan which had been incorporated into protein. This radioactivity was detected as follows: the pellets were extracted successively with 5 ml volumes of (v/v) propanol:ether (1:2), (v/v) propanol:ether: chloroform (2:2:1) and petroleum ether. The residual protein was dried under a stream of nitrogen and then was dissolved in 0.8 ml of 98% formic acid. To this mixture, 15 ml of scintillator was added (toluene:ethanol = 7:3 containing 100 mg of

dimethyl POPOP and 4 g of PPO per liter). Using this procedure, our results showed that less than 3% of the total radioactivity in the tissue was associated with the pellet following a 15 min incubation in the presence of 1.0 mM medium L-tryptophan.

F. Preparation of Tryptophan-Deficient Animals

The rats used in the tryptophan deficiency experiments weighed 130-140 g initially. They were fed solid semi-synthetic diets designated as control or tryptophan-deficient. Diets were prepared with the following ingredients (g per 3 kg batch): acid-hydrolysed, vitamin-free casein 660, corn starch 870, sucrose 900, partially hydrogenated fat (Crisco) 180, corn oil (Mazola) 60, powdered cellulose 60, and modified Rogers-Harper (1965) salt mixture 150. The composition of the salt mixture (percentage by weight) was as follows: ammonium molybdate 0.0025, calcium carbonate 29.290, calcium phosphate 0.430, magnesium sulfate 9.980, manganous sulfate 0.121, potassium iodide 0.0005, potassium phosphate 34.310, sodium chloride 25.181, sodium selenite 0.0015. Each 3 kg batch of feed contained the following vitamins (mg): biotin 6, folic acid 12, riboflavin, thiamine hydrochloride and pyridoxine hydrochloride 75 each, vitamin B_{12} 0.1% trituration in mannitol and menadione 150 each, calcium pantothenate 195, nicotinic acid (niacin) 300, inositol and p-aminobenzoic acid 330 each, and choline chloride 5010. The following amino acids were included in each batch

(grams): D,L-phenylalanine 26, and D,L-tyrosine 26. The control diet also contained 7 g of D,L-tryptophan and 55g of sucrose; deficient diet had no added tryptophan, but included an additional 62 g of sucrose over the basal diet. Finally, both batches of feed contained 0.9345 g ferric citrate, 60 mg cupric sulfate and 30 mg of zinc chloride. The animals were given food and water ad libitum. Body weights were recorded daily. At the end of twenty-eight days, the control animals had increased in weight to 255-280 g whereas the tryptophan-deficient animals weighed 100-110 g.

8. Calculations

A. Total Tissue Water

The total weight of rat cerebral cortex slices after incubation is comprised of (1) intracellular water originally present in the tissue, (2) extracellular water present initially in the tissue, (3) swelling fluid which the tissue acquires during incubation and (4) tissue solids (dry weight). Thus, the total tissue water was calculated as follows:

Total tissue water (µl) = Swollen tissue weight (mg)-Dry Weight (mg)
(after incubation) (after incubation)

(1)

B. Inulin Space

Inulin space is calculated from the amount of radioactive inulin in the slice in relation to the amount of radioactive inulin in the incubation medium in the following manner:

$$\text{Inulin space (\% initial fresh weight)} = \frac{\text{Total tissue counts per min}}{\text{Medium counts per min per ml}} \cdot \frac{10^5}{\text{Initial fresh weight (mg)}} \quad (2)$$

$$\text{Aerobic inulin space Corrected for tissue swelling (\% initial fresh weight)} = \text{Inulin space (\% initial fresh weight)} - \text{swelling fluid (\% initial fresh weight)} \quad (3)$$

C. Non-Inulin Space

$$\text{Non-inulin space (\% initial fresh weight)} = \text{Total tissue water (\% initial fresh weight)} - \text{inulin space (\% initial fresh weight)} \quad (4)$$

D. Assumptions Required for the Inulin Calculations

The above equations are based upon the following assumptions:

1. The specific gravity of rat cerebral cortex slices is 1.00.

2. Inulin is located essentially in the extracellular fluids of the tissue.

3. The extracellular compartment is continuous with the incubation medium so that the concentration of inulin in this space following incubation is the same as that of the incubation medium.

4. The non-inulin space obtained by subtracting the inulin space from this total tissue water is the true intracellular volume of the incubated tissue.

5. The tissue dry weights determined separately on sets of similarly prepared slices are comparable to the dry weight of slices which were actually incubated with inulin.

6. The swelling which occurs under aerobic conditions occurs only in the extracellular space (except during the incubation conditions listed in Tables 3 and 4).

E. Tryptophan Uptake

The total quantity of radioactive tryptophan in the intracellular water of slices of rat cerebral cortex immediately following incubation was usually calculated according to the equation:

$$\text{Total radioactivity/ml intracellular water} = \frac{\text{Total radioactivity in the tissue} - \left\{\text{cpm per ml medium} \left[\text{corrected inulin space, (ml)} + \text{swelling fluid (ml)}\right]\right\}}{\text{Non-inulin space (ml)}} \quad (5)$$

This equation was applicable to all the experiments detailed in this thesis, with the exception of the incubation conditions described in Tables 3 and 4.

When intracellular as well as extracellular swelling occurred during incubation (i.e., when the composition of the incubation medium was similar to one of those described in Tables 3 and 4), the following equation was used in calculations of tryptophan accumulation:

$$\text{Total radioactivity/ml intracellular water} = \frac{\text{Total radioactivity in the tissue} - \text{cpm per ml medium} \left[\text{inulin space (ml)}\right]}{\text{Non-inulin space (ml)}} \qquad (6)$$

It should be noted that tryptophan accumulation, as calculated by equations (5) and (6), has been computed on the basis of the initial specific radioactivity of the amino acid thus ignoring any contribution by the endogenous pool of the tissue.

F. Tryptophan Transport Ratio

$$\text{Tryptophan transport ratio} = \frac{\text{Total cpm per ml intracellular water}}{\text{cpm per ml medium}} \qquad (7)$$

RESULTS AND DISCUSSION

9. Inulin Space Studies

A. Preamble

Since this thesis was to be concerned with the accumulation of tryptophan by cerebral cortical cells, it was decided at the outset that the results obtained should be reported in μmoles of tryptophan accumulated per ml of intracellular water. The underlying reason for this choice was that cerebral cortex slices are known to undergo considerable swelling in the interstitial spaces when incubated *in vitro*. Since this space is for the most part continuous with the medium, a significant portion of the total tryptophan present in the slice following incubation is extracellular. Thus, to obtain an accurate picture of cellular tryptophan accumulation, it was necessary to correct for this extracellular tryptophan.

A number of comprehensive reports have been published over the last twenty years detailing the size of tissue spaces in slices of rat, mouse and cat brain. These excellent studies (which will be mentioned more specifically a little later on) have clearly shown that the size of the various tissue compartments depends to a great extent on the experimental methodology employed. Thus, the mode of tissue preparation, the type of

incubation medium, the experimental conditions and the marker used in these experiments all contribute to the size of the space values obtained. Furthermore, it is not always possible to correlate the results obtained with different markers using the same experimental conditions. Electron microscopy has been relatively unsuccessful in resolving these difficulties since the fixation techniques used in the preparation of electron micrographs are known to cause significant changes in the size of tissue spaces. Thus, to date, there are no absolute values available for the size of the intracellular space in rat brain cortex slices which could be used in the calculation of the cellular accumulation of tryptophan.

With the aforementioned reports in mind, it was concluded that an attempt must be made to ascertain the size of the various tissue compartments using our experimental conditions. For these studies inulin was chosen as the extracellular marker for a variety of reasons:

(1) It does not appear to penetrate cells as do sucrose, sodium, chloride and thiocyanate (Pappius, 1969; Goodman, Weiss and Alderdice, 1973).

(2) The distribution of inulin in cerebral tissues occurs by passive diffusion; there is no evidence of intracellular inulin uptake in vitro. (Varon and McIlwain, 1961; Pappius, Klatzo and Elliott, 1962; Bourke and Tower, 1966).

(3) Numerous investigators have used this extracellular marker and it was thought that this might permit interesting comparisons with our own data.

(4) Inulin is relatively inexpensive and readily available.

B. Inulin Space Determinations in Aerobic Conditions

Table 1 details the results obtained when slices of rat cerebral cortex were incubated aerobically for varying lengths of time in Krebs-Ringer-phosphate medium containing tracer amounts of inulin-{carboxyl-^{14}C}. From this table it can be seen that the inulin space attained constant values of around 58 µl/100 mg fresh weight of tissue, during incubation periods between 120 and 180 min. Similarly, the extent of tissue swelling and the size of the non-inulin space also reached constant values between 120-180 min. Since the inulin and non-inulin spaces remained constant during these 60 min, we concluded that the inulin had completely equilibrated with all the tissue fluids with which it was continuous. Thus, for rat cerebral cortex slices incubated aerobically the equilibrated inulin space using our experimental conditions was calculated to be 57.9 µl/100 mg fresh weight of tissue while the equilibrated non-inulin space was determined to be 62.1 µl/100 mg fresh weight of tissue. On the basis of these results, the studies outlined in Tables 2 and 3 were routinely of 180 min duration to insure complete equilibration of the marker.

Table 2 indicates the extent of swelling and the size of the inulin and non-inulin space in slices of rat cerebral

TABLE 1

Time Course of Swelling and Inulin Uptake into Rat Cerebral Cortex Slices Incubated Aerobically with No L-Tryptophan Present in the Incubation Medium*

Incubation Time (min)	Swelling µl/100 mg	Dry Weight mg/100 mg	Total Tissue Water µl/100 mg	Inulin Space µl/100 mg	Non-Inulin Space µl/100 mg	"Corrected" Inulin Space µl/100 mg
90	29.2 ± 2.9(5)	18.5 ± 0.8(18)	110.7	52.1 ± 2.4(5)	58.6	22.9
120	37.3 ± 5.0(6)	18.5 ± 0.8(18)	118.8	57.0 ± 2.3(6)	61.8	19.7
150	40.2 ± 3.5(5)	18.5 ± 0.8(18)	121.7	58.2 ± 1.1(5)	63.5	18.0
180	38.2 ± 3.0(5)	18.5 ± 0.8(18)	119.7	58.6 ± 2.3(5)	61.1	20.4

* Procedure as described in "Methods" Section 7C.
Tabulated data represent mean values ± S.E. (number of observations in parentheses), referred to the initial (fresh) weight of tissue.

TABLE 2

Swelling, Inulin Spaces and Non-inulin Spaces in Rat Cerebral Cortex Slices Incubated Aerobically in Media Containing 1.0 mM L-tryptophan but Varying in Ionic Composition*

Incubation Medium	Swelling µl/100 mg	Dry Weight mg/100 mg	Total Tissue Water µl/100 mg	Inulin Space µl/100 mg	Non-inulin Space µl/100 mg	"Corrected Inulin Space" µl/100 mg
1.0 mM L-Tryptophan	43.5±4.4(7)	18.5±0.8(18)	125.0	69.8±2.6(7)	55.2	26.3
" + 0.5% Inulin	35.7±2.2(4)	18.5±0.8(18)	117.2	61.6±4.4(4)	55.6	25.9
" + 1.0 mM L-DOPA	49.2±4.1(4)	18.5±0.8(18)	130.7	71.5±2.0(4)	59.2	22.3
" - K^+	20.2±9.3(3)	18.5±0.8(18)	101.7	42.1±4.4(3)	59.6	21.9
" + 10 mM Na^+	21.2±3.9(4)	18.5±0.8(18)	102.7	40.5±2.1(4)	62.2	19.3
" + 20 mM Na^+	20.6±3.5(4)	18.5±0.8(18)	102.1	40.4±1.8(4)	61.7	19.8
" + 60 mM Na^+	22.6±3.8(4)	18.5±0.8(18)	104.1	53.2±5.0(4)	50.9	30.6
" + 80 mM Na^+	22.0±4.0(4)	18.5±0.8(18)	103.5	34.9±2.7(4)	68.6	12.9
" + 100 mM Na^+	22.8±3.9(3)	18.5±0.8(18)	104.3	46.7±5.0(3)	57.6	23.9
" + 120 mM Na^+	17.1±1.8(4)	18.5±0.8(18)	98.6	39.0±1.5(4)	59.6	21.9
" + 5.5 mM Li^+	35.5±6.8(4)	18.5±0.8(18)	117.0	60.6±3.2(4)	56.4	25.1
" + 5.5 mM Cs^+	41.6±3.2(4)	18.5±0.8(18)	123.1	68.2±2.2(4)	54.9	26.6

* Procedure as described in "Methods" Section 7C.
All incubations were of 180 min duration at 37°C.
Isotonicity in media from which Na^+ or K^+ had been partially or completely removed was maintained by the addition of equimolar quantities of choline chloride.
To maintain isotonicity in the medium containing 5.5 mM Li^+ or 5.5 mM Cs^+, 5.5 mM Na^+ was removed.
Tabulated data represent mean values ±S.E. (number of observations in parentheses), referred to the initial (fresh) weight of tissue.

cortex which have been incubated in media to which 1.0 mM L-tryptophan has been added but which have also been altered in their ionic composition. The concentrations of the ions in each of these media were chosen so that the results might be applied to the calculation of tryptophan accumulation in later experiments where media of similar ionic composition were used. The usual Krebs-Ringer medium contained 145 mM NaCl and 5.5 mM KCl. In all the determinations listed in Table 2, when Na^+ or K^+ was removed from the medium, they were replaced by equimolar quantities of choline chloride.

The first two determinations in this table illustrate the effect which the addition of 1.0 mM L-tryptophan exerted on the sizes of the inulin space and of the non-inulin space in incubated cerebral cortex slices. For the first series of determinations only a tracer amount of inulin-{carboxyl-^{14}C} was added while in the second series of determinations, 0.5% carrier inulin as well as the radioactive inulin was present in the incubation medium. The results from both determinations were similar. The inulin space increased from the 57.9 µl/100 mg fresh weight of tissue value shown in Table 1 to the average value of 69.8 µl/100 mg fresh weight of tissue shown in Table 2. Correspondingly, the non-inulin space decreased from 62.1 to 55.2 µl/100 mg fresh weight of tissue. The 36-44% swelling which was observed was approximately the same as that observed when

the slices were incubated for the longer periods (120-180 min) listed in Table 1. Thus, tryptophan appears to decrease the size of the intracellular or non-inulin space. Also, carrier inulin had no effect on the space values, a finding which was also noted when Na^+ was absent from the medium and the slices were incubated at 4^oC (Table 3). These results agree with those reported by Varon et al. (1961) and by Cohen (1972), and were the underlying argument for the absence of carrier inulin in these studies.

The presence of 1.0 mM L-DOPA did not appear to alter the size of the inulin space or that of the non-inulin space significantly although the mean tissue swelling was increased.

Complete omission of K^+ and alterations in the Na^+ concentration of the media caused tissue swelling to decrease by approximately 50%. The inulin space values decreased by 20-48% while the non-inulin space values (except the 60 mM Na^+ value) remained within the range obtained in Table 1 for slices incubated for extended time periods.

The addition of 5.5 mM LiCl or 5.5 mM CsCl with the corresponding omission of 5.5 mM NaCl from medium containing 1.0 mM L-tryptophan resulted in inulin space values and non-inulin space values which were similar to those obtained under control conditions. Tissue swelling was also similar to that observed for slices incubated in control media. These results

indicate that replacement of Na^+ or K^+ by the addition of choline chloride has a pronounced effect on the amount of fluid absorbed by incubated brain slices. This effect is reflected only in the size of the inulin space.

Several investigators have reported that the presence or absence of certain compounds or ions in the incubation medium causes significant alterations to the size of the inulin and non-inulin spaces. Accordingly, during the course of this study, inulin determinations were made when the composition of the incubation medium contained these compounds or ions. The results are compiled in Table 3.

Incubation of the tissue slices at 37°C in a medium from which Na^+ had been completely omitted caused a large increase in the inulin space value, i.e., from 66.8 to 79.2 µl/100 mg fresh weight of tissue. Tissue swelling remained unchanged. If however, the same medium was used with slices incubated at 4°C, the inulin space decreased by 24-28%, tissue swelling was halved but the size of the non-inulin space remained at a constant value of 44-45 µl/100 mg fresh weight of tissue. As mentioned previously in discussing Table 2, the presence of carrier inulin did not alter these results.

Omission of 10 mM glucose from the incubation medium caused a considerable increase in the non-inulin space, from 55.3 to 86.5 µl/100 mg fresh weight of tissue, as well as a la

TABLE 3

Swelling, Inulin Spaces and Non-Inulin Spaces in Rat Cerebral Cortex Slices Incubated Aerobically in Media Known to Cause Significant Alterations in the Inulin and Non-Inulin Spaces*

Incubation Media		Swelling µl/100 mg	Dry Weight mg/100 mg	Total Tissue Water µl/100 mg	Inulin Space µl/100 mg	Non-Inulin Space µl/100 mg
1.0 mM L-Tryptophan	37°C**	40.6±3.0(11)	18.5±0.8(18)	122.1	66.8±2.5(11)	55.3
" - Na^+	37°C	43.0±3.4(4)	18.5±0.8(18)	124.5	79.2±3.6(4)	45.3
" - Na^+	4°C	22.7±2.4(4)	18.5±0.8(18)	104.2	60.5±2.1(4)	43.7
" -Na^+ + 0.5% Inulin	4°C	20.9±2.4(4)	18.5±0.8(18)	102.4	57.3±1.9(4)	45.1
" -10 mM Glucose	37°C	67.4±2.9(4)	18.5±0.8(18)	148.9	62.4±3.3(4)	86.5
" + 1.0 mM NaCN	37°C	32.5±5.0(4)	18.5±0.8(18)	114.0	71.2±2.0(4)	42.8
" + 1.0 mM L-Glutamate	37°C	51.9±2.4(4)	18.5±0.8(18)	133.4	82.2±1.0(4)	51.2
" + 145 mM K^+ + 5.5 mM Na^+	37°C	84.3±4.5(4)	18.5±0.8(18)	165.8	57.6±3.1(4)	108.2

* Procedure as described in "Methods" Section 7C.
All incubations were of 180 min duration at the temperature indicated.
Equimolar quantities of choline chloride were present in the media which contained no sodium.
Tabulated data represent mean values ± S.E. (number of observations in parentheses), referred to the initial (fresh) weight of tissue.

** This control value was calculated by combining the figures obtained from the first two studies listed in Table 2.

increase in tissue swelling; but the inulin space did not change. Thus, it appears that the absence of glucose from the incubation medium causes intracellular swelling in cortex slices.

Addition of 1.0 mM NaCN to the incubation medium decreased the size of the non-inulin space from 55.3 to 42.8 µl/100 mg fresh weight of tissue but this compound had little effect on either the size of the inulin space or on the amount of tissue swelling. The addition of 1.0 mM L-glutamic acid, however, had the opposite effect, i.e., it increased the inulin space from 66.8 to 82.2 µl/100 mg of fresh weight of tissue and the amount of tissue swelling from 40.6 to 51.9 µl/100 mg of fresh weight of tissue, but had little effect on the size of the non-inulin space (51.2 versus 55.3 µl/100 mg of fresh weight of tissue for the control medium). Complete reversal of the Na^+ and K^+ concentrations (i.e., 145 mM K^+ and 5.5 mM Na^+) caused a dramatic increase in the non-inulin space from 55.3 to 108.2 µl/100 mg fresh weight of tissue. Tissue swelling also increased substantially from 40.6 to 84.3 µl/100 mg fresh weight of tissue but the inulin space decreased only slightly from 66.8 to 57.6 µl/100 mg fresh weight of tissue. Thus, a high K^+ medium appears to cause intracellular swelling.

C. Inulin Space Determination in Anaerobic Conditions

Table 4 illustrates the results which were obtained

TABLE 4

Time Course of Swelling and Inulin Uptake into Rat Cerebral Cortex Slices Incubated Anaerobically with or without L-Tryptophan Present in the Incubation Medium*

Incubation Time (min)	Swelling µl/100 mg	Dry Weight mg/100 mg	Total Tissue Water µl/100 mg	Inulin Space µl/100 mg	Non -Inulin Space µl/100 mg
No Tryptophan					
30	33.0 ± 4.0(6)	18.5 ± 0.8(18)	114.5	45.5 ± 2.4(6)	69.0
60	57.3 ± 6.0(4)	18.5 ± 0.8(18)	138.8	50.9 ± 2.7(4)	87.9
90	59.9 ± 1.2(3)	18.5 ± 0.8(18)	141.4	54.2 ± 1.4(3)	87.2
120	66.6 ±11.6(4)	18.5 ± 0.8(18)	148.1	59.8 ± 1.8(4)	88.3
150	73.4 ±16.5(3)	18.5 ± 0.8(18)	154.9	68.2 ± 6.7(3)	86.7
+ 1.0 mM L-Tryptophan					
180	64.6 ± 5.6(4)	18.5 ± 0.8(18)	146.1	63.9 ± 2.3(4)	82.2

* Procedure as described in "Methods" Section 7C.
Tabulated data represent mean values ± S.E. (number of observations in parentheses), referred to the initial (fresh) weight of tissue.

when slices of rat cerebral cortex were incubated anaerobically for varying lengths of time in Krebs-Ringer phosphate medium containing tracer amounts of inulin-{carboxyl-^{14}C}. As in the case of the results shown in Table 1, the extent of tissue swelling and the size of the inulin space increase as the incubation period is prolonged. The size of the non-inulin space appears to reach a limiting value of 87-88 µl/100 mg fresh weight of tissue beyond 60 minutes of incubation whereas the inulin space continues to increase as the tissue swelling increases. The presence of 1.0 mM L-tryptophan in the incubation medium has little effect on the size of either the inulin or non-inulin space, and on the extent of tissue swelling.

D. Discussion

At the outset of this study, the wide variation in the extent of tissue swelling and in the size of the inulin space (Tables 1-4) were cause for great concern. Careful analysis of these results ultimately led to the use of equations 1-7 detailed in "Experimental" Sections 8A-F, for the calculation of tryptophan accumulation and transport. An attempt will be made here to outline the reasons underlying the use of these equations.

From the initial studies, and indeed, throughout all the experiments to be reported in this thesis, tissue swelling

was recognized as an important process to be taken into account. Even during incubation periods of 15 min or less, considerable swelling occurred. This swelling was not necessarily reproducible, i.e., four flasks could be incubated for the same time, in the same incubation medium and under the same experimental conditions, and yet tissue swelling could vary as much as 20%. Similarly, when cerebral cortex slices were incubated under the usual control assay conditions, i.e., for 15 min at 37°C in Krebs-Ringer phosphate medium containing 10 mM glucose and 1.0 mM L-tryptophan, swelling averaged 22.2% ± 0.9 (S.E.) (n=63) of the fresh weight of tissue. The range of these values extended from 6.5% to 42.6%. Clearly then, swelling was an important factor which had to be considered in all calculations of tryptophan uptake.

The studies of Pappius and Elliott (1956), Pappius Rosenfeld, Johnson and Elliott (1958), Varon et al. (1961), Pappius et al. (1962), Bourke et al. (1966) and Cohen, Blasberg, Levi and Lajtha (1968), have established that inulin is an extracellular marker which, only under abnormal incubation conditions (e.g., when retina is incubated in hypotonic medium), penetrates intracellularly (Ames, Isom and Nesbett, 1965). Some controversy exists, however, concerning the degree of penetration of inulin into the interstitial spaces of cerebral cortex slices incubated in vitro.

Pappius et al. (1956; 1962) in comparing in vitro studies of the sucrose, thiocyanate and inulin spaces of rat

cerebral cortex showed that the inulin space increases with the degree of tissue swelling and that the inulin space can be accounted for almost entirely by the water of swelling. They obtained inulin space values of 50-60% of the fresh weight of tissue when slices were incubated for more than 60 min in Krebs-Ringer bicarbonate medium containing 10 mM glucose. Since these investigators obtained sucrose and thiocyanate spaces which were considerably larger than the inulin space, they concluded that the molecular size of inulin prevents it from penetrating into the true extracellular fluids of brain tissue.

The results in Table 1 indicate that the size of the equilibrated inulin space obtained in this study was similar to the values obtained by Pappius et al. (1956; 1962). Also, during our incubations the degree of tissue swelling did increase as the time of incubation was prolonged. However, one significant difference exists between their studies and our own: in our study the inulin space was always much larger than the water of swelling. Indeed, we observed that if the water of swelling is subtracted from each of the inulin space values shown in Table 1, a space value which we designated the "corrected inulin space" is obtained which is relatively constant. The mean "corrected inulin space" calculated from the inulin space values in Table 1 is 20.3 µl/100 mg fresh weight of tissue and the range in these values is very narrow, from 18-23 µl/100 mg fresh weight of tissue. Similarly, when the same calculations are

made for the values listed in Table 2, a mean "corrected inulin space" of 23.0 µl/100 mg fresh weight of tissue is obtained. The range in these values from 13-31 µl/100 mg fresh weight of tissue, is much greater than that of Table 1. However, if one compares the considerable range in the inulin space (from 35-72 µl/100 mg fresh weight of tissue) and in the extent of tissue swelling (from 17-49 µl/100 mg fresh weight of tissue), the variation in the "corrected inulin space" is much less. We believe that the "corrected inulin space" represents the *in vivo* extracellular space of brain tissue plus the space occupied by any intracellular fluid which may have been released interstitially from damaged cells during the slicing process.

One assumption was made in calculating the "corrected inulin space" value: only extracellular swelling was presumed to occur during these incubations. This assumption was based on the findings of Pappius *et al.* (1956; 1962) who reported that during most aerobic incubations only extracellular swelling can be observed. The particular incubation conditions which have been noted to cause intracellular as well as extracellular swelling are listed separately in Tables 3 and 4 and will be discussed later.

The difference in the extent of inulin penetration into the fluids of cerebral cortex slices as reported by Pappius *et al.* (1956; 1962) and by this study probably lies in the

method of tissue preparation and incubation. Varon et al. (1961) have reported that fluid uptake by rat cerebral cortex slices is markedly dependent on the buffer used and especially on the time the slices are present in this buffer before optimal aerobic conditions are established. These investigators, as well as Pappius et al. (1956; 1958; 1962) and Bourke et al. (1966), have usually incubated their slices in bicarbonate-buffered media. Varon et al. (1961) have observed that tissue swelling is significantly increased when slices are exposed to bicarbonate medium equilibrated with air rather than one which has been equilibrated with 95% O_2/5% CO_2. They have also noted that although fluid uptake then increases, this "preparatory swelling" occurs in a compartment which is inaccessible to inulin. In our experiments the tissues were exposed to air for 10-12 min before immersion in phosphate-buffered medium and subsequent oxygenation. Hence one would expect, on the basis of the foregoing reports, to detect some difference between the results of these investigators and our own.

Temperature is another factor which appears to contribute significantly to the extent of inulin penetration into brain tissue fluids. Pappius et al. (1962) have reported that if tissues are prepared in the cold, a larger volume of the slice fluids is occupied by inulin than can be accounted for by the water of swelling. This observation is in agreement with the results detailed in Tables 1 and 2. More recent studies by

Cohen et al. (1968) have shown that there are two inulin compartments in mouse brain slices: a first inulin space which is permeable to inulin at 37°C but not at 0°C. According to these investigators, the first inulin space is much larger than the second inulin space. Furthermore, when slices are incubated in inulin-containing medium at 37°C and are subsequently cooled to 0°C, the inulin space is the same as when the slices are incubated at 37°C without cooling, i.e., in both instances the first inulin space is measured. On the basis of these studies Cohen (1972) defines the conventional non-inulin space to be the difference between the total tissue water at 37°C and the inulin space at 37°C. He believes that it is best to use the 37°C inulin space without correction for the second inulin space as the conventional, functional extracellular marker at 37°C.

In our studies, brain slices were prepared in a cold room set at 4°C, incubated at 37°C in an inulin-containing medium and were then immediately placed on ice before the incubation medium was withdrawn for scintillation counting. Thus, if compartmentation of inulin occurs in rat brain cortex slices in a similar manner to that reported by Cohen et al. (1968) for mouse brain slices, we can conclude that our inulin space values are comparable to the first inulin space of these investigators.

Calculation of tryptophan accumulation according to equation 5 outlined in "Experimental" Section 8.E is based on the results detailed in Tables 1 and 2. Thus, the mean non-inulin space value was calculated from all the data in these two

tables and was found to be 59.2 µl/100 mg fresh weight of tissue. Similarly, the "corrected" inulin space value was calculated to be 22.3 µl/100 mg fresh weight of tissue. These values were taken by us to represent the *in vitro* intracellular space and the *in vitro* extracellular space (corrected for swelling) of incubated brain tissue when placed in medium in which only extracellular swelling occurred. This method of calculation had the advantage of taking into account the individual swelling in each flask but assumed that this swelling was exclusively extracellular. Since Pappius *et al.* (1956) have noted that most aerobic swelling is extracellular and since Battistin, Grynbaum and Lajtha (1969) have reported that only the acidic amino acids glutamate and aspartate cause intracellular swelling in brain slices, it was thought that these assumptions were valid. Thus, for most of the studies reported in this thesis, equation 5 "Experimental" Section 8.E was used for the calculation of tryptophan uptake. However, when slices were incubated under metabolic conditions reported to alter the intracellular space (i.e., the media detailed in Tables 3 and 4), inulin space values were determined for each medium, and tryptophan accumulation was calculated according to equation 6, "Experimental" Section 8.E.

Tables 2 and 3 provide data on the effect of some alterations to the ionic composition of the Krebs-Ringer phosphage medium on the extent of tissue swelling and on the size of the inulin and non-inulin spaces. Replacement of the

5.5 mM KCl normally present in the medium by an equimolar quantity of choline chloride caused significant changes in the inulin space as well as in the swelling fluid. Thus, tissue swelling decreased 50%, the inulin space decreased by 37%, the "corrected" inulin space decreased by 16% while the non-inulin space increased by 8%. Similar results were obtained when varying concentrations of NaCl from 10-120 mM were present in the incubation media. However, replacement of 5.5 mM NaCl with an equimolar amount of LiCl or CsCl caused no significant change from control values in the extent of tissue swelling, or in the inulin space, non-inulin space and "corrected" inulin space values. These results indicated that a small quantity (5.5 mM) of LiCl or CsCl can replace NaCl in the medium without causing significant changes in the tissue spaces of incubated rat brain slices. If the same or larger amounts of choline chloride are used to replace either NaCl or KCl in the medium, tissue swelling and the inulin space decrease significantly but the non-inulin space and "corrected" inulin space values remain more constant. When NaCl is completely replaced by choline chloride (145 mM) in the medium as shown in Table 3 the non-inulin space decreased by 19% while the inulin space increased by a corresponding amount. When the temperature of this incubation medium was reduced from 37°C to 4°C, tissue swelling and the inulin space decreased significantly but the non-inulin space was not affected

any further. Thus our results indicate that complete replacement of NaCl by choline chloride caused intracellular shrinkage which was unaffected by the incubation temperature. As has been reported by Pappius et al., 1958, the lower incubation temperature partially prevented fluid absorption by the tissue.

The results obtained in this study are in partial agreement with those of other investigators. The difficulties encountered in comparing results can probably be attributed to the various experimental conditions used by each research group. Thus, Pappius et al. (1958) have shown that when rat cerebral cortex slices are incubated at 38°C in bicarbonate-buffered Krebs-Ringer medium in which NaCl has been completely replaced by choline chloride, tissue swelling remains unchanged. Similarly, Lahiri and Lajtha (1964) have also shown that swelling of mouse brain cerebral slices incubated at 37°C in Krebs-Ringer phosphate or tris-buffered media containing either 128 mM NaCl or 128 mM choline chloride is the same. Our results detailed in Table 3 show little change in the extent of tissue swelling when slices are incubated at 37°C in the presence or absence of sodium and thus are in good agreement with these investigators. Difficulties arise however in comparison of the inulin and non-inulin spaces. Pappius et al. (1958) reported that the non-sucrose space (32 µl/100 mg fresh weight of tissue) does not change when the medium contains either NaCl or choline chloride. Battistin et al. (1969) have shown that reduction

of the NaCl content of a tris-buffered Krebs-Ringer medium to 10 or 20 mM NaCl caused no significant change in the size of the inulin space (52.3% and 53.7% of the wet weight of tissue respectively) of mouse brain slices. Cohen et al. (1968) have reported similar inulin space results for mouse cerebrum slices incubated in sodium-free medium at 37°C. However, they have also observed a significant decrease in the non-inulin space. Our results show a decrease in the size of the non-inulin space in agreement with the results of Lajtha and coworkers for mouse brain slices but in disagreement with the sucrose studies of Pappius et al. (1958) for rat brain cortex. The significant increase in the size of the inulin space when slices are incubated in a sodium-free medium has not been reported before. The disagreement of this finding with the results of the investigators mentioned above may lie in the difference in initial manipulation of the brain tissue i.e., slicing at 4°C and "preparatory" swelling at 4°C.

Removal of the 10 mM glucose usually present in the Krebs-Ringer phosphate medium caused large increases in tissue swelling and in the non-inulin space as well as a slight decrease in the inulin space. Thus, these data indicated that incubation of brain tissue slices in a glucose-free medium causes intracellular swelling. Similar observations have been noted by Cohen et al. (1968) and by Gottesfeld and Elliott (1971).

Tissue swelling and the non-inulin space decreased when 1.0 mM NaCN was present in the incubation medium but there was no change in the inulin space. We concluded that using our experimental conditions, NaCN causes intracellular shrinkage. These results however are in contrast to those reported by Cohen et al. (1968) and by Battistin et al. (1969). Using mouse brain slices incubated in tris-buffered Krebs-Ringer medium, these investigators reported that the inulin space decreases by at least 6% in the presence of 1.0 mM NaCN. They concluded that NaCN causes intracellular swelling.

A similar discrepancy also exists between this group's study and our own on the effect of glutamate on fluid compartmentation. Cohen et al. (1968) and Battistin et al. (1969) reported decreased inulin space values when 2 mM glutamate was added to medium containing mouse brain slices. Our results, shown in Table 3, indicate that 1.0 mM glutamate increases the inulin space as well as the tissue swelling in rat brain slices and decreases the non-inulin space slightly. Pappius et al. (1956) observed increased tissue swelling and increased non-inulin space values with rat brain cortex slices incubated in Krebs-Ringer bicarbonate-buffered medium containing 5 mM glutamate. They noted that slices in this medium accumulate glutamate and K^+ rather rapidly and attribute the increase in the non-inulin space to intracellular swelling which occurs when

high K^+ concentrations are present inside the brain cells. At the present time we can offer no explanation as to the disparity between our results and those of the aforementioned investigators.

When slices were incubated in medium containing 145 mM KCl and 5.5 mM NaCl, tissue swelling doubled as did the size of the non-inulin space. The inulin space decreased by approximately 14%. Similar findings have been reported by Pappius et al. (1956) and are attributed to the intracellular influx of K^+.

The data of Table 4 describe the time course of inulin penetration under anaerobic conditions. As in aerobic incubation, tissue swelling increased with time. The inulin and non-inulin space values did not reach limiting values until incubation had been prolonged beyond 90 min. Addition of 1.0 mM L-tryptophan to the medium decreased the non-inulin space slightly, as under aerobic conditions. Anaerobiosis, however, caused greater tissue swelling than aerobiosis and it can readily be seen that the additional swelling was intracellular. Our results agree with those of Pappius et al. (1956). These investigators have shown that the non-sucrose and non-inulin spaces of rat brain slices incubated anaerobically are significantly increased over the space values obtained when slices are incubated aerobically and have concluded that anaerobiosis causes intracellular swelling.

To summarize briefly, we have shown that, in general,

the non-inulin space of cerebral cortex slices is 59.2 µl/100 mg fresh weight of tissue while the "corrected" inulin space (i.e., corrected for extracellular swelling) is 22.3 µl/100 mg fresh weight of tissue. The mean dry weight of these slices is 18.5 µl/100 mg fresh weight of tissue. It should be noted that there was no significant difference between the dry weight values of incubated or non-incubated slices — an observation in agreement with the findings of Pappius et al. (1956) and Bourke et al. (1966).

Pappius (1969) has recently reviewed the findings of numerous investigators concerning the size of the extracellular space of brain tissue of various species in vivo. She has reported that for rabbit brain, the chloride space corrected for intracellular chloride, is between 21 and 24%, and that the extracellular sodium space is 24%. She has noted that if the extracellular space of brain is assumed to be similar in composition to the cerebrospinal fluid of brain, then the sulfate space of cat, rat and dog brain tissue can be calculated to range from 16 to 24%. This investigator has also indicated that cisternal injection of radioactive sucrose resulted in a sucrose space for rabbit brain of 22.5% whereas intracisternal injection of labelled inulin gave inulin space values ranging from 18% for rabbit brain to 28% for cat brain and 34% for monkey brain. The thiocyanate space of cat cerebral cortex, after correction for intracellular penetration, ranged from 21 to 25%. Finally, measurements

of cortical impedance show that the extracellular space of rat, rabbit and cat brain tissue lies between 18 and 25%. Thus, the "corrected" inulin space determined by our study appears to be in agreement with the most recent in vivo measurements of extracellular space. Furthermore, the method of calculation generally used in the experiments reported here has the advantage of taking into particular account the swelling which occurs in each flask.

10. L-Tryptophan Transport: An Active Process

A. Preamble

The experiments described in this chapter were designed to elucidate the nature of tryptophan uptake by slices of rat cerebral cortex. Initially we determined the time interval during which tryptophan uptake is linear as well as the apparent Michaelis constant and maximal velocity of this uptake process. Varying the incubation conditions and the addition of several metabolic inhibitors to the incubation medium allowed us to investigate whether tryptophan accumulation is by an active mechanism. The effects of a number of ions (notably Na^+, K^+, Li^+, Mg^{2+} and Ca^{2+}) are reported. Some discussion is included regarding the suitability of the assay used to measure tryptophan uptake.

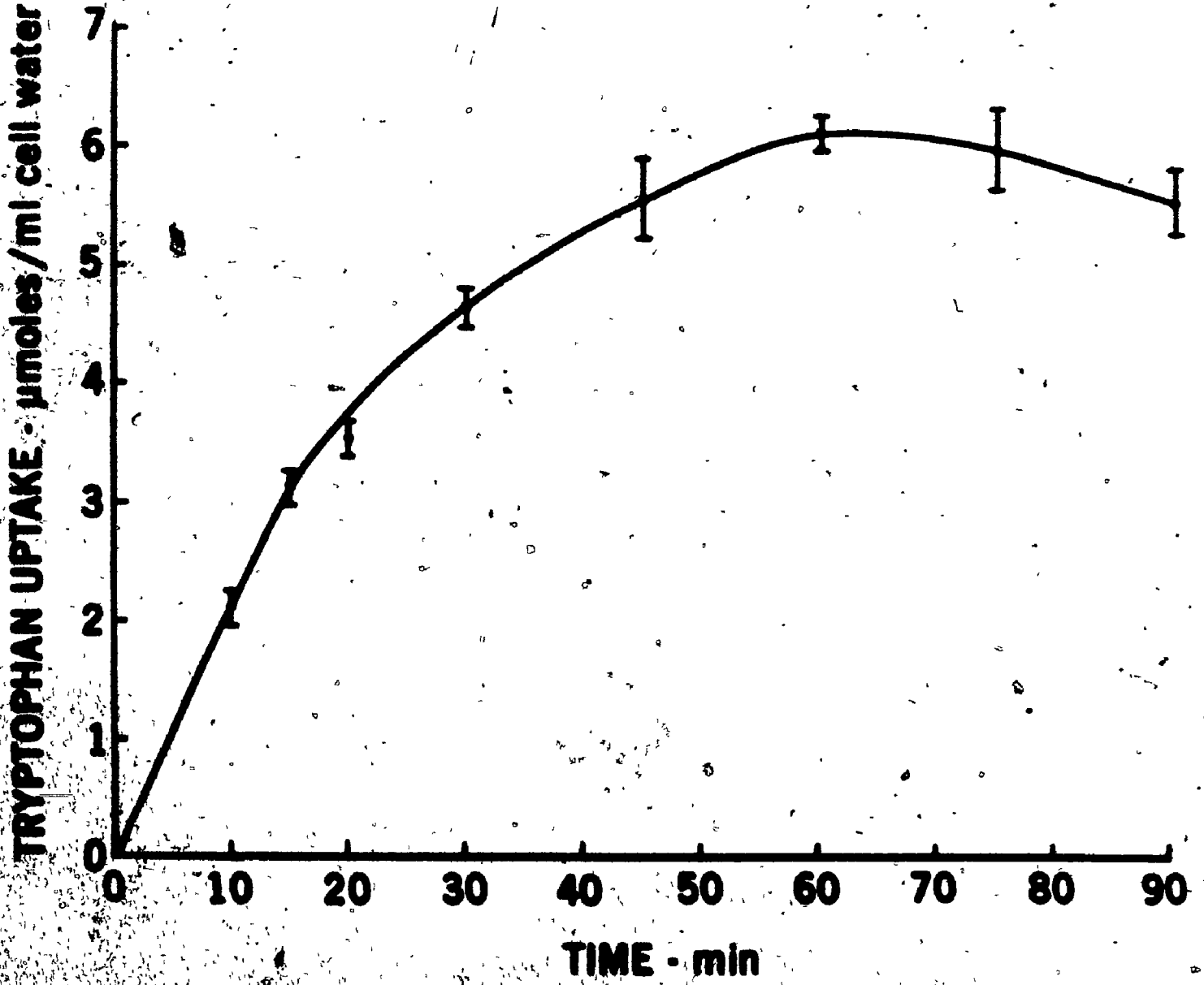
B. Time Course for L-Tryptophan Accumulation

The pattern of accumulation of 1.0 mM medium L-tryptophan by rat cerebral cortex slices during a 90 min incubation period is illustrated in Figure 3. There was a linear increase in tryptophan concentration during the first 15 min of incubation, after which accumulation increased more slowly. At 60 min, the tryptophan concentration had reached a maximal value of 6.10 μmoles/ml of cell water. For incubation periods longer than 60 min, tryptophan was accumulated to a slightly lesser extent

Figure 3

Time Course for the Accumulation of L-Tryptophan by Rat Cerebral Cortex Slices.

Points represent mean values ± S.E. for 4 determinations.


TRYPTOPHAN UPTAKE - μmoles/ml cell water
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
TIME - min

than the 60 min peak value probably because of disintegration of the tissue or metabolism of the amino acid or both.

C. Evidence Favoring Active Concentration of L-Tryptophan by Cortex Cells

The data in Table 5 present the results of experiments on the accumulation of L-tryptophan, 1.0 mM in the medium, by rat cerebral cortex slices during a 15 min incubation period. The accumulation is significantly decreased by anaerobiosis and/or by low incubation temperatures (6°C). The addition of some metabolic inhibitors, however, did not have a significant effect on tryptophan uptake by cortex cells. Thus, 1.0 mM iodoacetamide, an inhibitor of the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) reaction in the Embden-Meyerhof pathway of glucose metabolism or 1.0 mM NaCN or 0.2 mM 2,4-DNP, inhibitors of the Krebs cycle or 0.2 mM ouabain (Strophanthin G), a Na^+-K^+ ATPase (EC 3.6.1.3) inhibitor did not alter significantly tryptophan accumulation by the cortex slices.

The results detailed in Table 6 indicate that omission of 10 mM glucose from the incubation medium severely decreased the accumulation of 1.0 mM medium L-tryptophan by the slices. However, 10 mM fructose or 10 mM pyruvate supported tryptophan uptake to the same extent as 10 mM glucose. Addition of the biologically inactive D-isomer of lactic acid decreased

TABLE 5

Effect of Anaerobiosis, Temperature and of Some Metabolic Inhibitors on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Incubation Medium	Gas Phase in Flask	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	Complete	O_2	4	3.16 ± 0.21	
	Complete	N_2	4	1.58 ± 0.21	<0.01
2	Complete	O_2	3	2.73 ± 0.33	
	Complete 6°C	O_2	4	0.72 ± 0.13	<0.01
	Complete 6°C	N_2	4	0.74 ± 0.06	<0.001
3	Complete	O_2	4	3.08 ± 0.47	
	" + 1.0 mM iodoacetamide	O_2	4	2.95 ± 0.18	>0.05
4	Complete	O_2	6	2.93 ± 0.12	
	" + 1.0 mM NaCN	air	4	3.11 ± 0.18	>0.05
	" + 0.2 mM 2,4-DNP	O_2	4	2.83 ± 0.23	>0.05
	" + 0.2 mM ouabain	O_2	3	2.71 ± 0.35	>0.05

* Incubations were 15 min in duration and, unless otherwise stated, were conducted at 37°C. The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.
\+ Probability of difference from corresponding control.

TABLE 6

Effect of Various Energy Substrates on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Incubation Medium	Gas Phase in Flask	No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
1	Complete (i.e., + 10 mM glucose	O_2	4	2.93 ± 0.15	
	Glucose omitted	O_2	4	1.82 ± 0.15	<0.01
	Glucose omitted + 10 mM fructose	O_2	4	2.99 ± 0.09	>0.05
	Glucose omitted + 10 mM pyruvate	O_2	4	3.05 ± 0.22	>0.05
	Glucose omitted + 10 mM D-lactate	O_2	4	2.42 ± 0.12	<0.05
	Glucose omitted + 10 mM citrate	O_2	4	2.53 ± 0.09	<0.05
2	Complete (i.e., + 10 mM glucose)	O_2	4	3.16 ± 0.21	
	Glucose omitted + 10 mM succinate	O_2	4	2.33 ± 0.16	<0.05

* Incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen. The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
For each of the above determinations, cortex slices were pre-incubated in medium containing the same energy substrate as the medium in which L-tryptophan uptake was studied.

+ Probability of difference from corresponding control.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

accumulation significantly but not as much as the absence of glucose. Two intermediates in the Krebs cycle, citrate (10 mM) and succinate (10 mM) did aid tryptophan uptake but not as efficiently as glucose, fructose or pyruvate.

The data of Table 7 show that when the medium L-tryptophan concentration was increased to 5.0 mM and the slices were incubated for 15 min, results similar to those described in Table 5 were obtained. Thus, tryptophan accumulation was decreased significantly when slices were incubated in an anaerobic atmosphere but the addition of 1.0 mM NaCN or 0.2 mM ouabain to the incubation medium had no significant effect on tryptophan uptake.

In order to determine the importance of cellular metabolism on the accumulation of L-tryptophan by cerebral cortex slices, the following incubation conditions were applied: (i) the 145 mM NaCl usually present in the incubation medium was completely replaced with an equimolar quantity of choline chloride and (ii) the slices were incubated at 6°C. These conditions inhibited, almost entirely, the uptake of L-tryptophan when its concentration in the medium ranged from 0.06-1.0 mM (Table 8). The results in this table indicate that in 15 min 0.03-0.10 μmoles of tryptophan/ml of cell water entered the cells under conditions approximating passive diffusion when the L-tryptophan concentration in the medium varied from 0.06 to 1.0 mM. Thus, at the lowest medium L-tryptophan concentration

TABLE 7

Factors Affecting the Accumulation of 5.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Incubation Medium	Gas Phase in Flask	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
Complete	O_2	4	8.04 ± 0.49	—
"	N_2	4	4.09 ± 0.43	<0.001
" + 1.0 mM NaCN	air	3	6.73 ± 0.66	>0.05
" + 0.2 mM ouabain	O_2	4	7.15 ± 0.90	>0.05

* Incubations were 15 min in duration and were conducted at 37°C. The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B. Tabulated data represent mean values ± S.E.

\+ Probability of difference from corresponding control.

TABLE 8

Uptake of L-Tryptophan into Slices of Rat Cerebral Cortex Incubated Aerobically at 6°C in Media from Which Sodium Had Been Completely Removed*

Exptl. Set	Incubation Medium	Gas Phase in Flask	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	1.0 mM L-tryptophan	O_2	4	3.26 ± 0.23	
	Same except 0 mM Na^+, 6°C	air	3	0.10 ± 0.01	<0.001
2	0.5 mM L-tryptophan	O_2	4	2.15 ± 0.18	
	Same except 0 mM Na^+, 6°C	air	3	0.09 ± 0.02	<0.001
3	0.2 mM L-tryptophan	O_2	4	0.93 ± 0.03	
	Same except 0 mM Na^+, 6°C	air	4	0.06 ± 0.01	<0.001
4	0.06 mM L-tryptophan	O_2	4	0.37± 0.02	
	Same except 0 mM Na^+, 6°C	air	3	0.03 ± 0.00	<0.001

* Incubations were 15 min in duration and, unless otherwise stated, were conducted at 37°C. The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
Isotonicity in media from which Na^+ had been removed was maintained by the addition of equimolar quantities of choline chloride.
Tabulated data represent mean values ± S.E.

\+ Probability of difference from corresponding control.

studied (0.06 mM) the rate of uptake was one twelfth of that detected when slices are incubated under the standard conditions. At the highest medium L-tryptophan concentration studied, the rate of uptake was 33-fold less than that of the control. We concluded from these results that cellular metabolism plays an important role in the transport of L-tryptophan by cerebral cortex slices. It should be noted that wherever it is indicated in this thesis, the figures in Table 8 have been used to correct the observed tryptophan uptake values for the non-metabolic (i.e., passive) accumulation of L-tryptophan by the slices.

D. Kinetic Analysis of the L-Tryptophan Uptake Process

The relation between the rate of accumulation of L-tryptophan by slices of rat cerebral cortex and the concentration of that amino acid in the medium is shown in Figure 4. The plotted points in Figure 4 a indicate that the rate of accumulation was approximately linear with respect to the applied external concentration of L-tryptophan up to about 1.0 mM but thereafter the rate fell off gradually. Because the data as a whole seemed to conform to the characteristics of a hyperbola of the type $Y = aX^b$, a curve was fitted to this equation (Curve a Figure 4) and described by the expression:

$$V = 3.39S^{0.623} \qquad (8)$$

where V = the rate of accumulation of L-tryptophan (in μmoles of

Figure 4

Rate of Accumulation of L-Tryptophan by Slices of Rat Cerebral Cortex Plotted as a Function of Initial Concentration of the Amino Acid in the Medium.

On the ordinate, V = μmoles of L-tryptophan accumulated/ml of cell water in 15 min of incubation; on the abscissa, S = mmoles of L-tryptophan initially present per liter of medium. Points plotted in curve a represent mean values ± S.E. for 4 determinations (S.E. not shown for means near origin for sake of clarity). Curve b represents a transformation of the equation obtained for a plot of 1/V vs 1/S (for values of S between 0.2 and 2.0 mM) (see Section 10D equation 10). Curve c represents the arithmetical difference between curves a and b. See text for discussion.

the amino acid/ml of cell water per 15 min); S = the concentration of L-tryptophan initially present in the incubation medium (in mmoles/liter); and 3.39 and 0.623 are estimated constants. These constants were obtained mathematically as follows: log V was plotted as a function of log S and a straight line was fitted to these transformed data by the method of least squares. The intercept of the line on the ordinate was then log (a) and the slope of the straight line was the constant b.

Although the hyperbolic curve a of Figure 4 conforms to the observations over a wide range of concentrations of tryptophan, an attempt was made to assess the data by conventional analysis according to the method of Lineweaver and Burk (1934). Kinetic analysis of this kind, based upon the double-reciprocal plot illustrated in Figure 5, revealed a clear departure from linearity at the highest concentrations of tryptophan. If the points corresponding to these extremely high concentrations were neglected, the remaining points fitted the straight line described by:

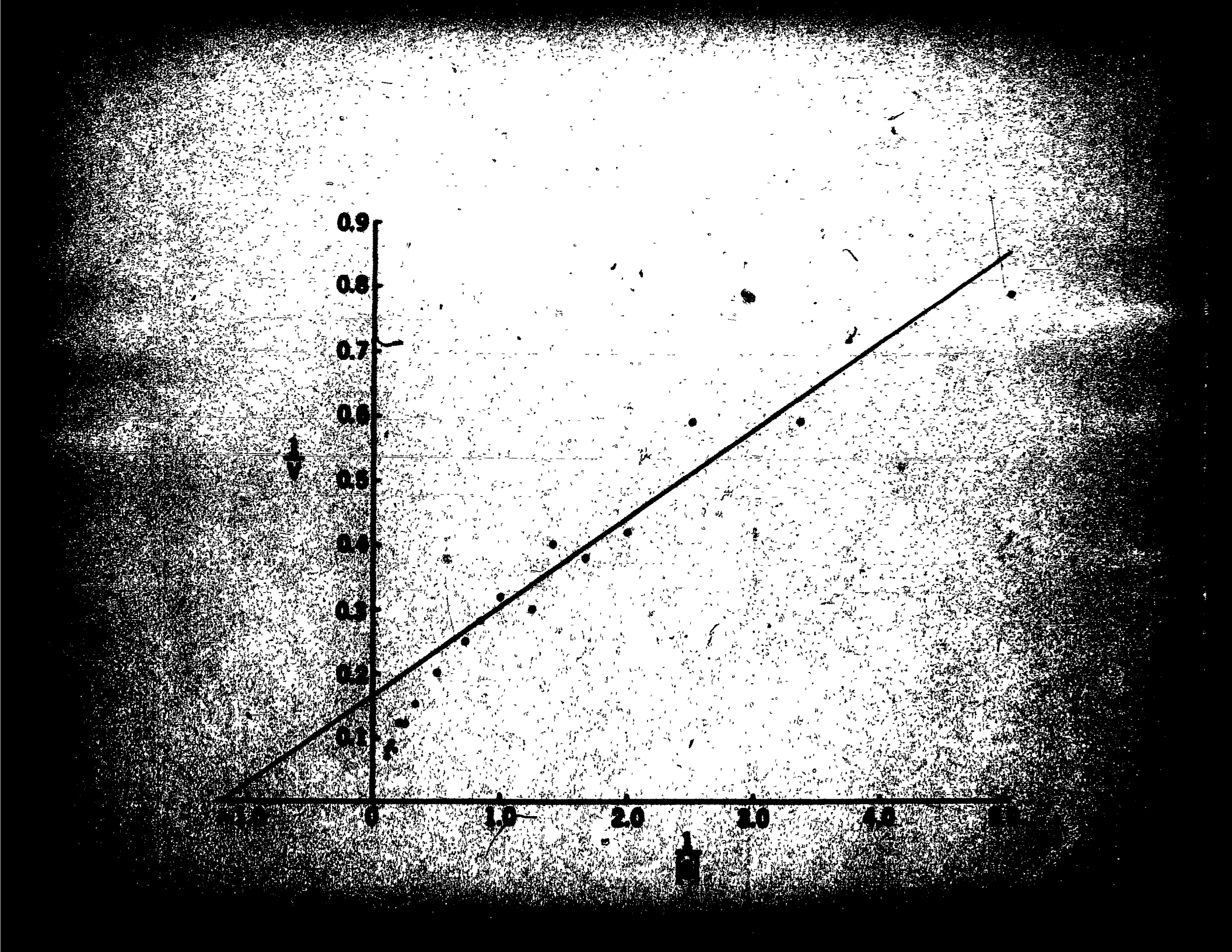
$$\frac{1}{V} = 0.166 + \frac{0.137}{S} = \frac{1}{6.032} + \frac{0.829}{6.032} \cdot \frac{1}{S} \qquad (9)$$

From this expression, the apparent Michaelis constant for the transport of L-tryptophan under our specific experimental conditions was 0.829 mM and the estimated maximal velocity of this system was 6.032 μmoles of L-tryptophan accumulated/ml of cell

Figure 5

Lineweaver-Burk Analysis of L-Tryptophan Uptake by Slices of Rat Cerebral Cortex.

On the ordinate V = μmoles of L-tryptophan accumulated/ml of cell water in 15 min of incubation; on the abscissa, S = mmoles of L-tryptophan initially present per liter of medium. Points represent mean values ± S.E. for 4 determinations.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0

water per 15 min of incubation. Transformation of the equation with these constants yielded the expression:

$$V = \frac{S}{0.166S + 0.137} = \frac{6.032\ S}{0.829 + S} \qquad (10)$$

represented by curve b in Figure 4.

For low concentrations of tryptophan in the incubation medium (i.e., up to about 1.0 mM), either of the hyperbolic equations described by curves a and b was consistent with the observed rates of accumulation of L-tryptophan. At higher levels of external L-tryptophan, the accumulation of this amino acid in the cell water was considerably greater than that accounted for by the assumptions implicit in Michaelis-Menten kinetics. The discrepancy between the two curves would be given by a complex expression representing the difference between equations (8) and (10) above. However, by empirically taking the arithmetical difference between the two curves a and b in Figure 4 a straight line resulted which by inspection fitted the equation:

$$V = -.90 + 0.98\ S, \qquad (11)$$

The slope of this line (0.98) represented the net change in the concentration of L-tryptophan accumulating in the cell water, under our specific experimental conditions, for a corresponding change in the concentration of the amino acid in the medium (both in μmoles/ml).

When the tryptophan transport ratio (i.e., the ratio of the tissue:medium concentration of L-tryptophan) was calculated from the data shown in Figure 4 and this ratio was then plotted versus the concentration of L-tryptophan in the incubation medium, the curve illustrated in Figure 6 was obtained. The highest ratio was 7.0 and this occurred when the medium concentration of L-tryptophan was 0.2 mM. However, the ratio decreased sharply as the medium L-tryptophan concentration was increased from 0.2-1.0 mM. Furthermore, medium concentrations greater than 2.0 mM yielded transport ratios which were slightly above 1.0. These results indicated that diffusion is an important process in tryptophan uptake by the cell when extracellular concentrations of L-tryptophan greater than 2.0 mM are presented to the cell membrane.

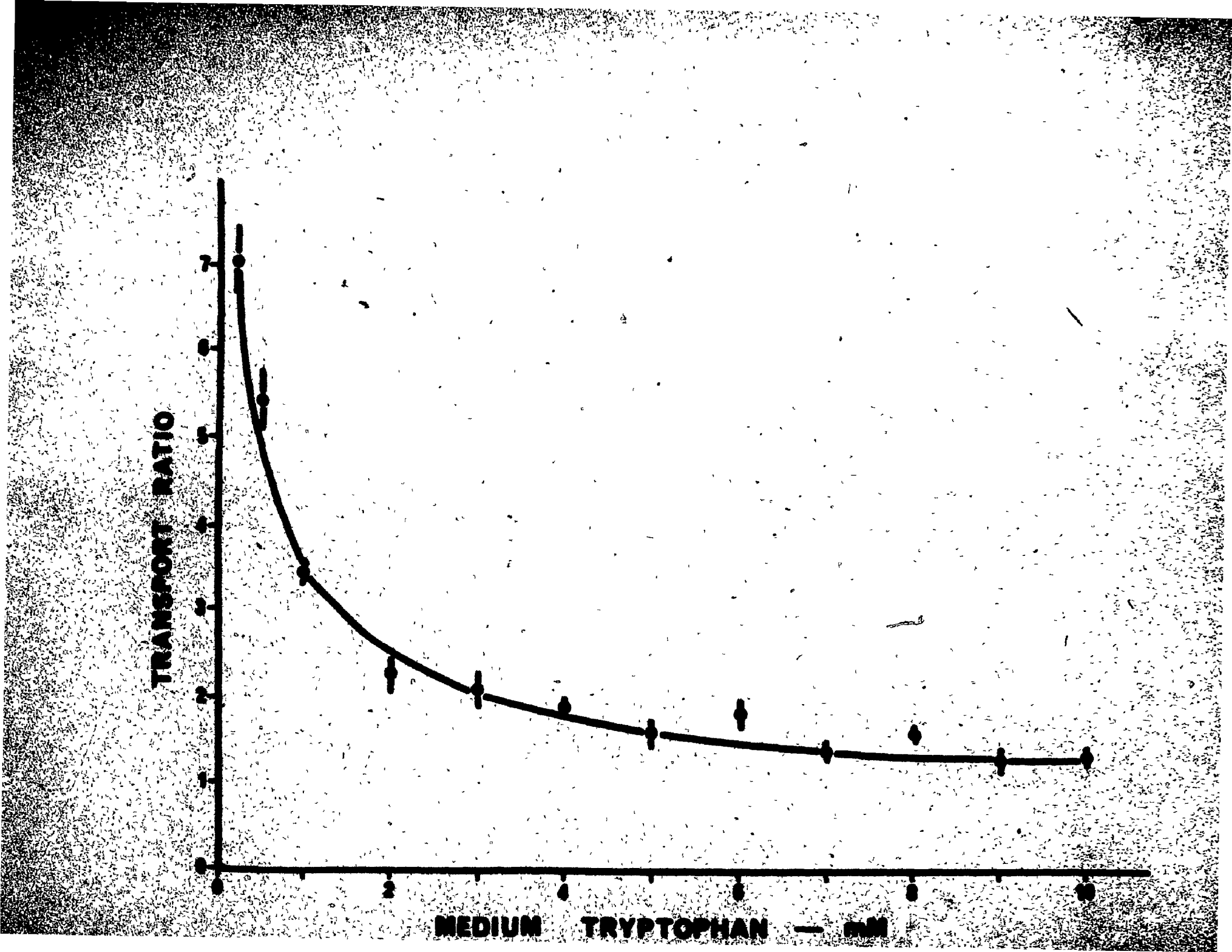
E. Observations Relating to the Assay Used in the Study of L-Trytpophan Accumulation

During the course of the studies reported in this thesis, we were concerned whether alterations to the experimental procedure described in "Methods" Sections 7A and 7B might significantly change the quantity of tryptophan accumulated by the cortex cells. Accordingly tryptophan uptake was measured following specific changes in the experimental procedure. The results of these studies are reported here:

Figure 6

The Ratio of L-Trytpophan Transport into Rat Cerebral Cortex Slices Plotted as a Function of the Initial Concentration of this Amino Acid in the Medium.

Points represent mean values ± S.E. for 4 determinations.


TRANSPORT RATIO
MEDIUM TRYPTOPHAN — mM

1. The amount of tissue present in each flask varied considerably. Although the amount of tryptophan taken up by the slices was proportional to the weight of the tissue used, the quantity of tryptophan accumulated per ml of cell water was remarkably constant at each medium tryptophan concentration. Thus, in 58 determinations with slices ranging in weight from 45-125 mg and incubated with 1.0 mM L-tryptophan for 15 min at 37°C, L-tryptophan uptake was 3.25 μmoles/ml cell water ± 0.07 (mean value ± S.E.).

2. In these experiments tissue slicing was usually performed in a cold room set at 4-6°C. Only superficial dorsal and lateral slices were used. If however, tissue slicing was performed at room temperature (21°C), there was no noticeable change in the tryptophan uptake values. We routinely sliced the tissue in the cold room to prevent rapid dehydration of the tissue which may alter the cells irreversibly and which decreases the ease with which the slices may be handled (Elliott, 1969).

   For a variety of reasons, only surface slices were used:

   (a) they are easier to obtain and tend to fragment less easily.
   (b) handling time is decreased when only the first slice is used.
   (c) there is only one cut surface per slice thereby decreasing the number of damaged cells per mg of tissue.
   (d) the use of only cerebral cortical matter is ensured since second slices of cortex frequently include subcortical white matter.

3. Figure 3 indicates that tryptophan accumulation is linear for the first 15 min of incubation when 1.0 mM L-tryptophan is present in the medium. Since we wished to study more closely the uptake of tryptophan within the linear portion of the time curve, we routinely used 15 min as the time interval for incubation of the tissue slices. The accumulation of 0.5 mM medium L-tryptophan is also linear within this time period. No other medium tryptophan concentrations were tested.

4. The uptake of 1.0 mM medium L-tryptophan at various incubation temperatures ranging from 22 to 50°C was investigated. We found that tryptophan was accumulated maximally around 40°C.

5. Usually 0.1 ml of L-tryptophan-{carboxyl-^{14}C} was added to 2.9 ml of the incubation medium. However there was no change in tryptophan accumulation if the volume of radioactive L-tryptophan added, was increased to 0.3 ml and the appropriate adjustments were made to the uptake calculations. Similarly, there was no change in the quantity of tryptophan accumulated by the cells when the volume of the incubation medium was doubled to 6.0 ml.

6. Following incubation, the slices were removed, placed on a porcelain filter disc and blotted carefully to remove excess medium. No attempt was made to wash the tissue. We found that such a step introduced a loss in tissue which caused greater error than failure to rinse the slices. These results agree with those of Cohen (1972).

7. We assumed that the radioactivity present in the tissue extracts following incubation represented tryptophan accumulated by the cells. This assumption was based on two factors:

   (a) we found that less than 3% of the total radioactivity in the tissue was incorporated into protein during a 15 min incubation period when slices were incubated with 1.0 mM L-tryptophan and then treated as described in "Methods", Section 7E.
   (b) the data of Barbosa, Herreros and Ojeda (1971) indicate that virtually all the radioactivity added as labelled L-tryptophan to rat brain cortex slices is accounted for (chromatographically) by that amino acid even after 60 min of incubation; Goldstein and Frenkel (1971) have also shown negligible conversion of tritiated L-tryptophan to serotonin by rat striatum and brain stem slices which had been incubated for 20 min at 37°C.

8. We found no difference in tryptophan uptake between animals which had been fasted for 24 hours and those which had been given free access to food.

F. Ions and L-Tryptophan Uptake

The effect of alterations to the ionic composition of the Krebs-Ringer phosphate incubation medium was examined (Tables 9 and 10). Complete replacement of NaCl by choline chloride caused a marked decrease in tryptophan accumulation. However, when NaCl was present in the incubation medium in concentrations greater than 20 mM, tryptophan uptake did not vary significantly from control values.

There was a significant decrease in tryptophan accumulated by the slices when the KCl, usually present in the incubation medium at 5.5 mM concentrations, was completely removed (Table 10). Increasing the KCl content of the medium to 10 mM caused no significant change in this accumulation. When the normal NaCl and KCl concentrations of the medium were reversed, however, tryptophan uptake decreased severely. Indeed, tryptophan accumulation under these conditions was much less than that observed when 5-10 mM NaCl plus 5.5 mM KCl were present in the medium (Table 9).

Neither calcium nor magnesium affected tryptophan accumulation (Table 10).

The results obtained when LiCl is added to the Krebs-Ringer phosphate incubation medium are detailed in Table 11. There was no significant change in tryptophan uptake when LiCl in concentrations ranging from 0.5-5.5 mM replaced NaCl in media

TABLE 9

Effect of Altering the Sodium Concentration of the Incubation Medium on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Sodium Composition of Incubation Medium	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	Complete(i.e., 145 mM Na^+)	4	3.26 ± 0.21	
	Same except (Na^+) = 0 mM	8	2.60 ± 0.14	<0.05
	5 mM	8	2.56 ± 0.14	<0.02
	10 mM	4	1.98 ± 0.33	<0.02
	15 mM	4	2.65 ± 0.20	>0.05
	20 mM	4	2.42 ± 0.23	<0.05
2	Complete (i.e., 145 mM Na^+)	4	3.14 ± 0.31	
	Same except (Na^+) = 25 mM	4	2.84 ± 0.21	>0.05
	30 mM	4	2.90 ± 0.05	>0.05
	35 mM	4	3.00 ± 0.23	>0.05
	40 mM	3	3.09 ± 0.19	>0.05
	45 mM	4	3.12 ± 0.17	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure and the composition of the incubation media are described in "Methods" Section 7A and 7B.
Isotonicity in media from which Na^+ had been removed was maintained by the addition of equimolar quantities of choline chloride.
It should be noted that the ionic composition of the pre-incubation media was identical to that of the incubation media in which L-tryptophan uptake was determined.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

+ Probability of difference from corresponding control.

TABLE 10

Effect of Altering the Potassium, Magnesium and Calcium Concentrations of the Incubation Medium on the Accumulation of 1.0 mM L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Composition of Incubation Medium	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	Complete	4	3.22 ± 0.40	
	Same except 0 mM K^+	3	2.18 ± 0.06	<0.05
2	Complete	4	2.95 ± 0.24	
	Same except 145 mM K^+;5.5 mM Na^+	4	0.70 ± 0.03	<0.001
	Same except 10 mM K^+;140.5 mM Na^+	4	3.45 ± 0.32	>0.05
3	Complete	6	3.49 ± 0.18	
	Same except 0 mM Ca^{2+}	8	3.46 ± 0.16	>0.05
4	Complete	4	3.50 ± 0.17	
	Same except 0 mM Mg^{2+}	4	3.60 ± 0.18	>0.05
5	Complete	4	3.16 ± 0.21	
	Same except 0 mM Mg^{2+}; 0 mM Ca^{2+}	4	3.04 ± 0.27	>0.05

* All incubations were 15 min duration and were conducted at 37°C in an atmosphere of 100% oxygen. The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
Isotonicity in the medium from which K^+ had been completely removed was maintained by the addition of equimolar quantities of choline chloride. When Ca^{++} and/or Mg^{++} were completely removed, no salt was added as replacement.
It should be noted that the ionic composition of the pre-incubation media was identical to that of the incubation media in which L-tryptophan uptake was determined.

\+ Probability of difference from corresponding control.

Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

TABLE 11

Effect of the Presence of Lithium in the Incubation Medium on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Composition of Incubation Medium	No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
		1.0 mM Medium L-Tryptophan		
1	Complete	3	2.69 ± 0.24	
	" + 5.5 mM Li^+ (replacing Na^+)	4	2.48 ± 0.18	>0.05
2	Complete	4	2.67 ± 0.32	
	" + 1.0 mM Li^+ (replacing Na^+)	4	2.62 ± 0.12	>0.05
		0.2 mM Medium L-Tryptophan		
3	Complete	4	0.91 ± 0.03	
	" + 0.5 mM Li^+ (replacing Na^+)	4	1.07 ± 0.10	>0.05
4	Complete	8	0.90 ± 0.02	
	" + 1.0 mM Li^+ (replacing Na^+)	12	0.83 ± 0.04	>0.05
5	Complete	4	0.91 ± 0.03	
	" + 5.5 mM Li^+ (replacing Na^+)	4	0.96 ± 0.06	>0.05
6	Complete	8	0.96 ± 0.03	
	" + 5.5 mM Li^+ (replacing K^+)	8	0.70 ± 0.05	<0.001
7	Complete	4	0.91 ± 0.03	
	" + 1.0 mM Li^+ + 4.5 mM K^+	4	0.77 ± 0.13	>0.05

TABLE 11 (Cont'd)

Exptl. Set	Composition of Incubation Medium	No of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
8	Complete	4	1.01 ± 0.05	
	" minus K^+	4	0.85 ± 0.05	>0.05
		0.06 mM Medium L-Tryptophan		
9	Complete	3	0.37 ± 0.02	
	" + 0.5 mM Li^+ (replacing Na^+)	4	0.33 ± 0.03	>0.05
	" + 1.0 mM Li^+ (replacing Na^+)	4	0.37 ± 0.01	>0.05
	"+5.5 mM Li^+ (replacing Na+)	3	0.38 ± 0.01	>0.05
	" + 5.5 mM Li^+ (replacing K^+)	8	0.30 ± 0.02	>0.05
	" + 1.0 mM Li^+ + 4.5 mM K^+	4	0.31 ± 0.03	>0.05
	" minus K^+	4	0.29 ± 0.02	<0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
It should be noted that the ionic composition of the pre-incubation media was identical to that of the incubation media in which L-tryptophan uptake was determined.
Isotonicity of the media from which K^+ had been completely removed was maintained by the addition of equimolar quantities of choline chloride.
LiCl replaced equimolar quantities of NaCl or KCl as indicated.
For the media containing 1.0 mM Li^+ and 4.5 mM K^+, the LiCl replaced 1.0 mM KCl.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.
+ Probability of difference from corresponding control.

containing 0.06-1.0 mM L-tryptophan. There was a significant decrease in the uptake of 0.20 mM medium L-tryptophan only when 5.5 mM LiCl replaced 5.5 mM KCl. This decrease was attributed to the absence of KCl rather than the presence of LiCl since there were decreases in tryptophan accumulation from media containing 0.06 or 0.20 mM L-tryptophan but containing no KCl.

Table 12 indicates that cesium, rubidium and thallium have no effect on the uptake of either 0.2 mM or 1.0 mM medium L-tryptophan by rat brain cortex slices.

G. Discussion

Brain slices actively concentrate L-tryptophan. This phenomenon, first reported by Joanny et al. (1968) has been confirmed by the present studies. Optimal conditions for the study of the effects of various amino acids, tryptophan metabolites and tryptophan analogs on the initial velocity of uptake of L-tryptophan by slices of rat cerebral cortex (to be discussed subsequently) were determined from the data illustrated in Figures 3 and 4 and from the observations reported in Section 10E. Thus, from Figure 3 we noted that incubation periods of 15 min duration would be within the linear range for accumulation of 1.0 mM concentrations of L-tryptophan from the medium. Since the results shown in Figure 4 indicated that tryptophan accumulation tended to increase with increasing concentrations of tryptophan

TABLE 12

Effect of the Presence of Cesium, Rubidium and Thallium in the Incubation Medium on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Composition of Incubation Medium	No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
		1.0 mM Medium L-Tryptophan		
1	Complete	4	2.80 ± 0.06	
	" + 5.5 mM Cs^+ (replacing Na^+)	4	2.75 ± 0.31	>0.05
	" + 5.5 mM Rb^+ (replacing Na^+)	4	2.86 ± 0.09	>0.05
		0.2 mM Medium L-Tryptophan		
2	Complete	4	0.91 ± 0.03	
	" + 1.0 mM Cs^+ (replacing Na^+)	4	0.73 ± 0.09	>0.05
	" + 1.0 mM Rb^+ (replacing Na^+)	4	0.86 ± 0.04	>0.05
	" + 1.0 mM Tl^+ (replacing Na^+)	4	0.91 ± 0.10	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure and the composition of the incubation media are described in "Methods" Sections 7A and 7B.
It should be noted that the ionic composition of the pre-incubation media was identical to that of the incubation media in which L-tryptophan uptake was determined.
CsCl, RbCl or TlCl replaced equimolar quantities of NaCl as indicated.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

+ Probability of difference from corresponding control.

in the incubation medium and that this rate of increase fell off with concentrations of tryptophan greater than 3.0 mM, we chose 1.0 mM as the medium tryptophan concentration to be used during most of our studies. The results reported in Section 10 B confirmed that the various steps of the experimental procedure were satisfactory.

When the tryptophan transport ratio was plotted against concentrations of L-tryptophan in the medium as illustrated in Figure 6, it was noted that beyond 3.0 mM medium L-tryptophan, the transport ratio was approximately 1.5. Similar results have been reported by Barbosa, Joanny and Corriol (1970) for the transport of L-tryptophan by rat cerebral cortex slices which had been incubated for 60 min at 37°C. Guroff et al. (1961) have also observed that L-tyrosine uptake by rat cerebral cortex cells is similar to the data shown in Figure 6. They suggested that with high medium tyrosine concentrations, the carrier mechanism for tyrosine was saturated by substrate.

Analysis of the data in Figure 5 showed that tryptophan accumulation could be formally represented by Michaelis-Menten kinetics when the concentration of the amino acid in the medium was less than 3.0 mM L-tryptophan. Greater concentrations showed a departure from linearity when analysed according to the method of Lineweaver and Burk (1934). We have been able to describe this accumulation in terms of two well-known kinetic processes, active transport and simple diffusion represented by

curves b and c (Figure 4) respectively. Experimentally these two processes are described by the non-saturable hyperbolic curve a in Figure 4 which illustrates the quantity of tryptophan accumulated at various medium L-tryptophan concentrations from 0.2 to 10.0 mM. Mathematically, we have described these processes by the sum of equations (10) and (11) in Section 10 D which are now combined in the following expression:

$$V = \frac{6.032\ S}{0.829 + S} + 0.98\ S - 0.90 \qquad (12)$$

This equation (12) is formally identical to the expression which Vahvelainen and Oja (1972) have adduced for L-tryptophan accumulation in rat brain slices except in regard to the constant (-0.90). We interpret our observations to mean that cerebral cortex cells have an active carrier mechanism which becomes saturated with concentrations of L-tryptophan near 1.0-3.0 mM in the medium. With higher concentrations, the slices accumulate L-tryptophan by diffusion pari passu with increased concentrations in the medium as indicated by the unit diffusion constant (slope of curve c in Figure 4, cf. equation 11) at these concentrations.

We have also noted that curve c in Figure 4 (generated from the arithmetical difference between curves a and b, Figure 4) becomes evident only with medium concentrations of L-tryptophan greater than 1.0 mM. We have concluded that at the lower medium

L-tryptophan concentrations, between 0.2 and 3.0 mM, there is diffusion of tryptophan from the cells because the intracellular concentration of the amino acid is much higher than that of the medium (cf., the tissue:medium concentration ratios shown in Figure 6). This outward diffusion of tryptophan is small and negative in value and is not readily detectable when the cells are actively concentrating L-tryptophan. With higher medium concentrations of L-tryptophan however, the carrier becomes saturated, the tissue:medium concentration ratio decreases towards one and the net flow of tryptophan diffusion is then directed into the cells thus making the diffusion values positive in sign.

In our experiments, the maximal velocity (Vmax) of the saturable transport process was calculated to be 6.032 μmoles of L-tryptophan accumulated/ml of cell water in 15 min of incubation; the apparent Michaelis constant (Km) was calculated to be 0.829 mM and the diffusion constant (Kd) was found to be 0.98 μmoles/ml cell water/15 min/mM. Although Joanny et al. (1968) and Barbosa et al. (1970) have studied the uptake of L-tryptophan by rat cerebral cortex slices at various L-tryptophan concentrations, they have never published Vmax or Km values. Vahvelainen et al. (1971) have reported that the Vmax for the intracellular accumulation of L-tryptophan by rat brain slices is 38 nmoles/min/g wet weight of tissue while the Km value is 0.30 mM and the Kd is 32 nmoles/min/g wet weight of tissue/mM. The discrepancy between

these results and our own may be partially attributed to the inulin space determinations. For their calculations, Vahvelainen et al. (1971) used an inulin space correction factor which they determined following only 5 min of incubation. By their own admission, this correction factor may have been inaccurate and hence led to a lower Vmax value. Our inulin studies certainly indicate this since 5 min is much too short an incubation period to allow for complete equilibration of the inulin.

Two research groups have studied the kinetic characteristics of L-tryptophan transport by rat brain synaptosomes. Grahame-Smith and Parfitt (1970) have reported that tryptophan uptake in synaptosomes prepared from whole brain show saturable Michaelis-Menten kinetics. They have calculated that the Vmax of this process is 225 nmoles of tryptophan accumulated/5 min/g fresh weight of brain and that the Km is 1.0 mM. More recently, Belin and Rujol (1972a; 1972b; 1973) have shown that there are three transport systems for tryptophan accumulation by synaptosomes prepared from rat brain mesencephalon: (1) a low affinity system seen at medium L-tryptophan concentrations between 0.1-2.0 mM which has a Vmax between 7.4-8.3 nmoles/mg protein/min and a Km between 0.7-1.8 mM, (2) an intermediate affinity system seen at medium concentrations between 0.05-0.25 mM which has a Vmax of 1.23 nmoles/mg protein/min and a Km of 0.08 mM, and (3) a high affinity system seen at medium concentrations between

2-50 μM which has a Vmax of 0.35-0.54 nmoles/mg protein/min and a Km of 6-14 μM. (The figures obtained by these investigators vary depending upon the range in medium concentration studied. We have included the upper and lower limits reported for each system.) In our studies, for concentrations between 0.2-10.0 mM L-tryptophan in the medium we have been unable to detect more than one affinity system for tryptophan. However, it is possible that more affinity systems could be detected in cerebral cortex if medium concentrations of L-tryptophan lower than 0.2 mM were presented to the cell membrane.

Our findings that the active carrier mechanism for tryptophan transport might be saturated when 1-3 mM L-tryptophan is present has interesting physiological significance. The normal concentration of tryptophan in rat blood is about 0.05 mM and in brain 0.01-0.02 mmoles/kg. (Schurr et al., 1950; Sourkes et al., 1970; Grahame-Smith, 1971). Thus, the concentration of this essential amino acid in rat plasma would have to exceed by 20 to 60-fold its normal level before the carrier mechanism would become saturated.

The results detailed in Tables 5-10 indicate that optimal accumulation of L-tryptophan by rat cerebral cortex slices is an energy-requiring process. It requires an energy-yielding substrate and is inhibited by anaerobiosis and/or low incubation temperatures as well as the omission of Na^+ or K^+ ions from the

incubation medium.

The metabolic inhibitors used in this study (iodoacetamide, NaCN and 2,4-DNP, cf. Tables 5 and 7) did not inhibit the transport of 1.0 mM or 5.0 mM L-tryptophan significantly. These results indicate that the levels of energy-rich phosphate compounds present in the tissue slices were probably sufficient to support tryptophan accumulation by the cortex cells even after the inhibitors had acted upon their target enzymes. It should be noted that for the inhibitor studies, the tissue slices were pre-incubated for 10 min at 37°C in medium containing 10 mM glucose before the inhibitor and radioactive L-tryptophan were added. Thus, the slices may have been able to produce sufficient ATP in this time interval so that tryptophan could be accumulated optimally despite the presence of an inhibitor. The results obtained by Barbosa et al. (1968) seem to support this hypothesis. These investigators reported that 1.0 mM NaCN and 0.2 mM 2,4-DNP inhibited tryptophan uptake severely when rat brain cortex slices were incubated for 40 min at 38°C. However, in their studies the slices were incubated in the absence of glucose and there was no pre-incubation period in the presence of glucose.

Tables 5 and 7 indicate that 0.2 mM ouabain had no effect on the accumulation of either 1.0 mM or 5.0 mM medium L-tryptophan. We expected that ouabain would inhibit tryptophan transport since this compound is known to be an effective Na^+-K^+

ATPase inhibitor. However, it is possible that ouabain requires a longer incubation period (than 15 min) to combine with the membrane ATPase and, presumably, to inhibit indirectly amino acid accumulation.

We have shown that the carbohydrates, glucose fructose, and pyruvate are the most useful energy substrates for the transport of L-tryptophan by cortex slices and that the Krebs cycle intermediates, citrate and succinate are markedly less efficient (Table 6). Barbosa et al. (1968) have reported observations which are similar to these results. They have found that 20 mM pyruvate, 20 mM lactate and 10 mM glucose stimulated tryptophan transport optimally when slices were incubated for 40 min at 38°C. These investigators have also noted that with their experimental conditions, slices incubated with 20 mM concentrations of α-ketoglutarate, succinate and fumarate accumulated the same amount of tryptophan as did slices which were incubated in a glucose-free medium.

Transport of L-tryptophan by cerebral cortex slices appears to be Na^+ and K^+ dependent. The results in Tables 8,9 and 10 indicate that the presence of Na^+ and K^+ ions in the incubation medium is necessary for tryptophan to be accumulated maximally. The presence of Ca^{2+} and/or Mg^{2+} in the incubation medium does not appear to be essential. Barbosa et al. (1970) have reported that the omission of Na^+ alters the apparent Km for tryptophan transport in rat cerebral cortex slices. They

have not, however, published these Km values. Grahame-Smith et al. (1970) have reported that when the concentration of Na^+ ions in the medium was varied from 0 to 112 mM by osmotic replacement with choline chloride or sucrose, there was no apparent effect on L-tryptophan uptake by rat brain synaptosomes prepared from whole brain. Removal of K^+, Ca^{2+} or Mg^{2+} also had no effect on tryptophan uptake. Our results show that incubation of tissue slices in a Na^+ free medium at 37°C for 15 min caused a significant decrease in the quantity of L-tryptophan accumulated when compared to slices which had been incubated under control conditions (e.g. 145 mM Na^+; cf. Table 9). However, tryptophan uptake did return to control values when concentrations of Na^+ greater than 25 mM were present in the medium.

Clinical interest in Li^+ and more recently in Cs^+ and Rb^+ for the treatment of endogenous depression led us to investigate whether these ions might effect tryptophan uptake by rat brain cortex slices. The results in Table 11 indicate that when 0.5-5.5 mM LiCl replaced equimolar quantities of NaCl in the incubation medium, there was no significant effect on the accumulation of 0.06-1.0 mM medium L-tryptophan. Also, Li^+ was not an effective replacement for K^+. The data detailed in Table 12 show that Cs^+, Rb^+ and Tl^+ had no effect on the uptake of either 0.2 or 1.0 mM medium L-tryptophan. Tl^+ was included in this study because it is a monovalent cation with an atomic radius which is

intermediate in size to that of Na^+ and K^+. Thus the low medium concentrations of Li^+, Cs^+, Rb^+ and Tl^+ have no effect on the tryptophan carrier in cerebral cortex cells.

Studies by Tagliamonte, Tagliamonte, Perez-Cruet, Stern and Gessa (1971) and Perez-Cruet and Eichelman (1972) showed that intraperitoneal administration of lithium carbonate (60 mg/kg twice a day for 5 days) or of cesium chloride (2 mg/kg twice a day for 5 days) to rats caused substantial increases in brain tryptophan and serotonin concentrations. They suggested that Li^+ and Cs^+ facilitate the entry of tryptophan into brain cells. Schubert (1973) also has reported that rat brain tryptophan increases significantly when animals are fed for seven days on a diet containing LiCl. A recent study by Knapp and Mandell (1973) provides an explanation for the discrepancy between our findings and those of the aforementioned investigators. Knapp et al. (1973) observed that synaptosomes prepared from rat brain striata exhibit two affinity systems for tryptophan uptake: a low affinity uptake system with a Km of 3.3 mM and a high affinity uptake system with a Km of 0.55 μM. They reported that synaptosomes prepared from animals which had received subcutaneous injections of LiCl (5-10 meg/kg for 5, 10 or 21 days) augmented the uptake of tryptophan by the high affinity system but that there was no effect on the low affinity uptake system. Our tryptophan accumulation studies have centered on a carrier mechanism which has an

apparent Km of 0.83 mM. Thus our tryptophan carrier system fits into the category designated by Knapp et al. (1973) to be a low affinity uptake system. Hence our negative findings regarding the effect of Li^+, Cs^+, Rb^+ and Tl^+ on tryptophan transport by cortex slices would seem to be in agreement with the results of these investigators.

11. Amino Acids, Tryptophan Metabolites and Tryptophan Analogs: Effects of These Compounds on Tryptophan Accumulation

A. Preamble

Numerous reports have been published concerning the various amino acid transport mechanisms which are present in brain tissue (cf. Introduction, Section 5E). These studies have included observations regarding the effect of tryptophan on the accumulation of some amino acids. To date, however, very little research has centered on the nature of tryptophan uptake by brain tissue or on the effect of other amino acids on this accumulation. Accordingly, the experiments described in this chapter, were designed to determine which, if any, of the commonly occurring amino acids might affect tryptophan transport. We were also interested in detecting whether metabolites derived from the pathways of tryptophan metabolism might alter tryptophan concentration by cerebral cortex cells. Finally, we were fortunate to have, in our laboratory, some tryptophan analogs which are not generally obtainable commercially. We used these compounds in an attempt to elucidate the structural specificity of the tryptophan transport carrier.

B. Results and Discussion

(i) Amino acids

L-phenylalanine D,L-p-chlorophenylalanine,

L-leucine, L-isoleucine and L-valine in 1.0 mM medium concentrations inhibited the accumulation of 1.0 mM medium L-tryptophan (Table 13). The effects of two other aromatic amino acids, L-tyrosine and L-DOPA however, were more variable (Tables 14 and 15).

L-tyrosine decreased tryptophan uptake significantly when 1.0 mM of this amino acid was present in incubation media containing 0.2 mM L-tryptophan whereas lower tyrosine concentrations (0.2 mM and 0.5 mM) in similar media had no effect. There was no change in tryptophan accumulation when 0.2 mM L-tyrosine was incubated with 0.5 mM L-tryptophan but higher tyrosine concentrations, i.e., 0.5 mM and 1.0 mM significantly inhibited the uptake of 0.5 mM medium L-tryptophan. Finally, 0.2 mM and 0.5 mM L-tyrosine had no effect on the accumulation of 1.0 mM medium L-tryptophan. However, 1.0 mM L-tyrosine significantly decreased the uptake of 1.0 mM medium L-tryptophan.

The action of L-DOPA on tryptophan accumulation was more specific. Concentrations of L-DOPA less than 1.0 mM in the medium did not alter the uptake of either 0.06 or 1.0 mM L-tryptophan when compared to control values. Higher L-DOPA concentrations between (1.0-4.0 mM) however, decreased significantly the uptake of L-tryptophan initially 0.2 mM, 0.5 mM or 1.0 mM in the medium. The data of Table 16 illustrates how the remaining amino acids (in 1.0 mM concentrations): glycine, L-alanine, L-threonine, L-serine, L-histidine, L-proline, L-hydroxyproline, L-cysteine, L-methionine, GABA, L-glutamic acid, L-glutamine, L-lysine and L-arginine had no effect on the uptake of 1.0 mM

TABLE 13

Amino Acids Which Inhibited the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Additions	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	None	6	3.12 ± 0.27	
	L-phenylalanine	8	2.00 ± 0.14	<0.01
2	None	7	3.12 ± 0.22	
	D,L-p-chlorophenylalanine	8	1.81 ± 0.15	<0.001
3	None	6	3.31 ± 0.18	
	L-leucine	8	2.35 ± 0.14	<0.01
4	None	4	2.65 ± 0.13	
	L-isoleucine	4	1.79 ± 0.03	<0.001
5	None	8	2.93 ± 0.22	
	L-valine	7	1.62 ± 0.20	<0.001

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
The incubation media contained 1.0 mM L-tryptophan as well as 1.0 mM concentrations of the amino acid tested.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

TABLE 14

Effect of L-Tyrosine on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
L-Tryptophan	L-Tyrosine			
0.2	-	4	0.93 ± 0.03	
0.2	0.2	4	0.94 ± 0.06	>0.05
0.2	0.5	4	0.90 ± 0.05	>0.05
0.2	1.0	4	0.66 ± 0.01	<0.001
0.5	-	4	2.15 ± 0.18	
0.5	0.2	4	1.96 ± 0.29	>0.05
0.5	0.5	4	1.16 ± 0.10	<0.01
0.5	1.0	4	1.30 ± 0.13	<0.01
1.0	-	8	3.07 ± 0.17	
1.0	0.2	3	2.53 ± 0.08	>0.05
1.0	0.5	4	2.86 ± 0.10	>0.05
1.0	1.0	8	2.63 ± 0.07	<0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

TABLE 15

Effect of L-DOPA on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	L-DOPA			
0.06	-	4	0.42 ± 0.05	
0.06	0.02	4	0.35 ± 0.08	>0.05
0.2	-	4	0.93 ± 0.03	
0.2	1.0	4	0.61 ± 0.05	<0.01
0.2	1.5	4	0.58 ± 0.06	<0.01
0.2	2.0	4	0.53 ± 0.01	<0.001
0.2	4.0	4	0.33 ± 0.02	<0.001
0.5	-	4	2.15 ± 0.18	
0.5	1.0	4	1.28 ± 0.13	<0.01
0.5	1.5	4	1.47 ± 0.14	<0.05
0.5	2.0	4	1.17 ± 0.13	<0.01
0.5	4.0	4	1.10 ± 0.11	<0.01
1.0	-	4	3.45 ± 0.16	
1.0	0.2	4	3.27 ± 0.29	>0.05
1.0	0.5	4	2.95 ± 0.47	>0.05
1.0	1.0	4	2.75 ± 0.16	<0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

+ Probability of difference from corresponding control.

TABLE 16

Amino Acids Which Had No Effect on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Additions	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	None	7	3.09 ± 0.12	
	Glycine	8	3.43 ± 0.29	>0.05
2	None	3	3.00 ± 0.10	
	L-alanine	4	3.36 ± 0.48	>0.05
3	None	8	3.08 ± 0.19	
	L-threonine	7	3.02 ± 0.25	>0.05
4	None	4	3.29 ± 0.16	
	L-serine	4	3.62 ± 0.22	>0.05
	L-histidine	4	3.36 ± 0.26	>0.05
	L-hydroxyproline	4	3.60 ± 0.26	>0.05
	L-cysteine	4	3.84 ± 0.38	>0.05
	L-methionine	4	3.25 ± 0.14	>0.05
5	None	3	3.00 ± 0.10	
	L-proline	4	2.86 ± 0.48	>0.05
6	None	4	3.22 ± 0.40	
	0.5 mM GABA	4	2.65 ± 0.21	>0.05
	None	8	3.00 ± 0.22	
	GABA	8	3.25 ± 0.29	>0.05
7	None	8	2.93 ± 0.22	
	L-glutamic acid	8	2.34 ± 0.39	>0.05
8	None	8	2.72 ± 0.11	
	L-glutamine	8	2.79 ± 0.22	>0.05
9	None	4	2.65 ± 0.13	
	L-lysine	4	2.71 ± 0.10	>0.05
	[illegible]ine	4	2.70 ± 0.20	

TABLE 16 (Cont'd)

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
The incubation media contained 1.0 mM L-tryptophan as well as 1.0 mM concentrations of the amino acid tested (except as noted for 0.5 mM GABA).
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.
+ Probability of difference from corresponding control.

medium L-tryptophan by rat cerebral cortex slices.

The results detailed in Tables 13-16 confirm the findings of Blasberg et al. (1965; 1966). These investigators reported that there are at least six carrier systems in mouse brain which are responsible for the transport of almost all the naturally-occurring amino acids into brain cells. They showed that one of these carrier systems transported the large neutral amino acids. All the amino acids which we found to inhibit tryptophan uptake (i.e., phenylalanine, p-chlorophenylalanine, tyrosine, DOPA, leucine, isoleucine and valine) belong to this category. Our studies, however, were different from those of Blasberg et al. (1965; 1966): they investigated the effect of tryptophan on the transport of a number of amino acids, whereas we have studied the effect of some amino acids on tryptophan transport. Thus, our results confirm the claim that tryptophan is indeed transported into brain cells by the carrier of the large neutral amino acids.

Several reports have indicated that phenylalanine and tyrosine inhibit the uptake of tryptophan by rat brain cortex slices (Barbosa et al., 1970; Green and Curzon, 1970; Barbosa et al., 1971). Furthermore, Grahame-Smith et al. (1970) have shown that phenylalanine is a competitive inhibitor of tryptophan transport into synaptosomal preparations of rat brain. They have reported that the Km for synaptosomal tryptophan transport is

1.0 mM and that the Ki for phenylalanine in this system is 0.16 mM. Joanny, Natali, Hillman and Corriol (1973) have recently published Km and Vmax values for L-phenylalanine and L-tyrosine influx into rat brain cortex slices. These investigators report that the Km for L-phenylalanine is 0.86 mM and that the Vmax is 0.64 μmoles/ml tissue water/min whereas the Km for L-tyrosine is 1.64 mM and the Vmax is 0.98 μmoles/ml tissue water/min. One can recall that we have determined that the Km for tryptophan transport is 0.83 mM and that the Vmax is 0.40 μmoles/ml cell water/min (Chapter 10 Section D). The similarity between our results and those of Joanny et al. (1973) and of Grahame-Smith et al. (1970) provide additional evidence indicating that the transport of tryptophan, phenylalanine and tyrosine into rat brain cerebral cortex slices is by the same carrier.

Aside from the aromatic amino acids (phenylalanine, p-chlorophenylalanine, tyrosine and DOPA) we found that the only other amino acids which inhibited tryptophan uptake were leucine, isoleucine and valine (Table 13). These results are interesting in view of the findings reported by other investigators. In vivo studies by Peng, Gubin, Harper, Vavich and Kemmerer (1973) have shown that when rats are force-fed a high (5%) tryptophan diet, there is a marked decrease (within 3 hours) in brain concentrations of isoleucine, valine, histidine, methionine, phenylalanine and tyrosine. Similar results have been reported by Fernstrom,

Larin and Wurtman (1973). They have been able to demonstrate that the tryptophan concentration of rat brain depends not only upon the plasma tryptophan concentration but also upon the plasma concentration of the other neutral amino acids (leucine, isoleucine, valine, phenylalanine and tyrosine). They reported that brain tryptophan concentrations remained unchanged when rats were fed a synthetic diet which contained the other neutral amino acids in addition to tryptophan. Rats which were permitted to consume a diet which contained only tryptophan showed significant increases in plasma and brain tryptophan. The relationship between brain tryptophan concentration and plasma levels of the other large neutral amino acids is attributed by this group to the carrier mechanism (elucidated by Blasberg et al., 1965; 1966) which mediates tryptophan transport into brain. Thus, our results are consistent with their observations.

The concept of a neutral amino acid carrier in brain tissue which mediates tryptophan transport into cells and which is affected by other large neutral amino acids is of physiological importance. As we have mentioned previously (cf. Introduction, Section 2), brain tissue utilizes tryptophan for protein synthesis and for the formation of serotonin. However, it has been shown that the concentration of tryptophan in brain is usually lower than that required to saturate tryptophan-5-hydroxylase, the enzyme believed to be the rate-limiting step in serotonin

synthesis. The endogenous pool of brain tryptophan is known to be the smallest pool of all the amino acids pools which are available for protein synthesis in brain tissue. If tryptophan uptake (and consequently the concentration of brain tryptophan) is affected by the plasma concentrations of the other large neutral amino acids as our data and that of Blasberg et al. (1965; 1966), Fernstrom et al. (1973), Peng et al. (1973) indicate, then the mechanism for tryptophan transport into brain may indeed be a rate-controlling step in the formation of brain proteins and of the postulated neurotransmitter, serotonin.

Several other amino acids have been reported to inhibit tryptophan uptake. Grahame-Smith et al. (1970) observed that methionine and glycine inhibited tryptophan transport into rat brain synaptosomes. Green et al. (1970) noted that 1.0 mM L-alanine caused 22% inhibition in the uptake of 6.0 μM medium L-tryptophan by rat brain slices following a 50 min incubation at 37°C. We have been unable to correlate our results with those of these investigators.

Histidine has also been observed to inhibit tryptophan accumulation. Using rat brain slices, Neame (1964) showed that L-tryptophan was a potent inhibitor of L-histidine transport. Barbosa et al. (1971) confirmed this finding. They reported that 1.0 mM medium L-histidine inhibits the uptake of 1.0 mM medium L-tryptophan and vice versa. However both of these

histidine studies involved incubation of the slices for 50-60 min. Since Grahame-Smith et al. (1970) have shown that histidine is a potent stimulator of tryptophan efflux from synaptosomes, it is possible that histidine acts on the efflux of tryptophan from cortex slices and that its effect can be detected only following prolonged incubation. If this explanation is correct, then the lack of effect of histidine on the initial influx of tryptophan shown in Table 16 would be clarified.

Table 15 indicates that high concentrations of L-DOPA ranging from 1.0-4.0 mM in the medium inhibit the uptake of 0.2-1.0 mM L-tryptophan by rat brain cortex slices. Analysis of these results according to the methods of Lineweaver and Burk (1934) and Dixon and Webb (1964) showed that action of L-DOPA on the tryptophan carrier did not conform to simple competitive kinetics. The data do however suggest that high concentrations of this amino acid can interact with the mechanism which transports tryptophan into brain cortex cells. Two in vivo studies have shown that L-DOPA does not affect brain tryptophan concentrations. Karobath, Diaz and Huttunen (1971) found only a slight decrease in rat brain tryptophan following a single intraperitoneal injection (100-200 mg/kg) of L-DOPA. Liu, Ambani and Van Woert (1972) showed that there was no significant change in rat brain tryptophan after chronic treatment (subcutaneous injection of 1000 mg/kg for 1 week) with L-DOPA. Our results

agree with these findings since they indicate that the plasma concentration of L-DOPA would have to be quite high before tryptophan transport into brain is affected. Our data are of clinical interest because of the current use of this amino acid in the treatment of Parkinson's disease. According to Tyce, Muenter and Owen (1970) peak concentrations of DOPA reached 2-3 hours after an oral therapeutic dose are in the range of 1-4 µg/ml of plasma. With such concentrations of L-DOPA in the incubation medium, viz. 0.02 mM, there was no inhibition of tryptophan accumulation. Thus, the normal therapeutic dose of L-DOPA may not affect tryptophan uptake into human brain cells.

Yoshida, Namba, Kaniike and Imaizumi (1963a) and Yoshida, Kiniike and Namba (1963b) have published extensive studies of L-DOPA transport into guinea pig brain. The characteristics of this transport bear startling similarity to tryptophan transport into rat brain cortex slices as elucidated in this thesis. Yoshida et al. (1963a, 1963b) have found that L-DOPA is actively concentrated by guinea pig cortex slices with medium concentrations of L-DOPA below 2.0 mM. They have observed that the active carrier mechanism for L-DOPA becomes saturated when the L-DOPA is between 2.0 and 3.0 mM. These investigators showed that with L-DOPA concentrations greater than 3.0 mM, the amino acid enters the cortex cells by passive diffusion. Furthermore, they reported that mannose, fructose,

and pyruvate as well as glucose stimulate L-DOPA transport but that Krebs cycle intermediates (α-ketoglutarate and succinate) are ineffective. Omission of Na^+ and K^+ decreased L-DOPA accumulation but this accumulation returned to control values when 80 mM Na^+ and 6.0 mM K^+ were present in the incubation medium. Finally, of the amino acids tested, they found that only L-phenylalanine and L-tyrosine inhibited the uptake of L-DOPA. The similarity between these results and our results which are outlined in Tables 5-16 provide additional evidence of L-DOPA transport by the same carrier as that for tryptophan.

(ii) Tryptophan Metabolites and Tryptophan Analogs

Table 17 illustrates the effect which L-5-hydroxy-tryptophan, an amino acid and tryptophan metabolite, exerted on the accumulation of L-tryptophan by rat cerebral cortex slices. The only significant inhibition observed was by 0.5 mM L-5-hydroxy-tryptophan in the presence of 0.2 mM L-tryptophan.

Schanberg and Giarman (1960), Schanberg (1963) and Smith (1963) have studied 5-hydroxytryptophan transport into rat and dog brain slices. These investigators have shown that 5-hydroxytryptophan is actively concentrated by brain slices from both species. Schanberg (1963) has reported that 1.0 mM L-phenylalanine, L-tyrosine, L-DOPA or L-tryptophan inhibits the uptake of 0.1 mM 5-hydroxy-D,L-tryptophan by rat brain cortex slices. Inhibition by these amino acids ranged from 25-45%

TABLE 17

Effect of L-5-Hydroxytryptophan on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentrations (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	L-5-Hydroxytryptophan			
0.06	-	4	0.37 ± 0.02	
0.06	0.02	4	0.36 ± 0.02	>0.05
0.06	0.05	4	0.33 ± 0.01	>0.05
0.2	-	4	1.01 ± 0.10	
0.2	0.05	4	1.03 ± 0.09	>0.05
0.2	0.5	4	0.72 ± 0.03	<0.05
1.0	-	3	3.00 ± 0.10	
1.0	1.0	4	3.39 ± 0.15	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.
\+ Probability of difference from corresponding control.

of the control uptake values. He has also observed that 0.1-1.0 mM concentrations of D-tryptophan have little effect on the uptake of 0.05-0.2 mM 5-hydroxy-D,L-tryptophan. Smith (1963) has published similar findings. He has reported that L-phenylalanine and α-methyl-D,L-DOPA are competitive inhibitors of 5-hydroxy-D,L-tryptophan uptake by rat brain slices. Smith (1963) also noted that L-tryptophan, L-tyrosine, L-histidine, L-glutamic acid and D,L-DOPA interfered with 5-hydroxytryptophan accumulation. Both Schanberg (1963) and Smith (1963) have noted that high concentrations of L-tryptophan (of the order of 1.0 mM) were required to inhibit the uptake of 0.05 mM-0.2 mM 5-hydroxy-D,L-tryptophan. These data and the results in Table 17 indicate that 5-hydroxytryptophan is probably transported into brain cells by the same carrier system as tryptophan.

Of the tryptophan metabolites tested, L-5-hydroxytryptophan was the only one with an intact pyrrole ring (cf. Figure 2) which inhibited tryptophan uptake. Thus, the results summarized in Table 18 show that 1.0 mM concentrations of 5-hydroxytryptamine, tryptamine, 5-hydroxyindoleacetic acid, indole-3-acetic acid or D,L-3-indolelactic acid did not significantly increase or decrease the uptake of 1.0 mM medium L-tryptophan.

Metabolites from the pyrrolase pathway of tryptophan degradation (cf. Figure 1) also did not exert a significant

TABLE 18

Effect of Some Tryptophan Metabolites (With the Pyrrole Ring Intact) on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Additions	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	None	6	3.03 ± 0.31	
	5-Hydroxytryptamine (creatinine sulfate complex)	8	3.06 ± 0.15	>0.05
	Creatinine sulfate	4	3.09 ± 0.18	>0.05
2	None	3	3.00 ± 0.10	
	5-Hydroxyindoleacetic Acid	3	3.17 ± 0.22	>0.05
3	None	8	3.18 ± 0.23	
	Tryptamine	8	2.74 ± 0.15	>0.05
4	None	4	3.16 ± 0.18	
	Indole-3-acetic acid	4	3.06 ± 0.53	>0.05
5	None	4	3.08 ± 0.47	
	D,L-3-Indolelactic acid	4	3.01 ± 0.16	>0.05

* All incubations were 15 min in duration and were conducted in an atmosphere of 100% oxygen. The procedure is described in "Methods" Sections 7A and 7B. The incubation media contained 1.0 mM L-tryptophan as well as 1.0 mM concentrations of the metabolite tested. Indole-3-acetic acid was dissolved in Krebs-Ringer phosphate medium to which ethanol had been added to a final concentration of 3% (v/v). Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

effect on tryptophan transport into cerebral cortex slices (Tables 19-22) with the exception of L-kynurenine. Thus, 1.0 mM concentrations of N'formyl-L-kynurenine, xanthurenic acid, quinolinic acid, quinaldic acid, picolinic acid or nicotinic acid as well as 0.5 mM kynurenic acid had no effect on the uptake of 1.0 mM medium L-tryptophan by cerebral cortex slices (Table 19). More detailed studies of the tryptophan metabolites, L-kynurenine, L-3-hydroxykynurenine and anthranilic acid showed that only L-kynurenine affected tryptophan transport (Tables 20-22). Thus, 0.5 and 1.0 mM medium concentrations of L-kynurenine significantly decreased the accumulation of 1.0 mM L-tryptophan from the medium (Table 20). However, when the L-tryptophan concentration was lowered to 0.2 mM and 0.5 mM, respectively, L-kynurnenine had little effect. Only 1.0 mM L-kynurenine inhibited the uptake of 0.5 mM L-tryptophan.

Green et al. (1970) reported that 1.0 mM concentrations of L-kynurenine and of L-3-hydroxykynurenine caused 35% inhibition in the uptake of 6 μM L-tryptophan into rat brain slices. They also observed that 3-hydroxyanthranilic acid, anthranilic acid, xanthurenic acid and quinolinic acid had no effect on tryptophan uptake. Our results are in agreement with those of these investigators except with regard to L-3-hydroxykynurenine.

The effects of various tryptophan analogs on the

TABLE 19

Effect of Some Metabolites of the Pyrrolase Pathway of Tryptophan Degradation on the Accumulation of 1.0 mM Medium L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Exptl. Set	Additions	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
1	None	4	3.16 ± 0.21	
	N'formyl-L-kynurenine	4	2.88 ± 0.25	>0.05
2	None	4	3.29 ± 0.16	
	Kynurenic acid (0.5 mM)	4	2.92 ± 0.15	>0.05
	Xanthurenic acid	4	3.38 ± 0.30	>0.05
3	None	4	3.08 ± 0.47	
	Quinolinic acid	4	3.04 ± 0.08	>0.05
	Quinaldic acid	3	2.33 ± 0.43	>0.05
	Picolinic acid	4	2.98 ± 0.17	>0.05
	Nicotinic acid	4	2.93 ± 0.17	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
The incubation media contained 1.0 mM L-tryptophan as well as 1.0 mM concentrations of the metabolite tested (except as noted for 0.5 mM kynurenic acid).
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected to diffusion.

\+ Probability of difference from corresponding control.

TABLE 20

Effect of L-Kynurenine on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	L-Kynurenine			
0.2	-	4	0.93 ± 0.03	
0.2	0.05	4	0.95 ± 0.14	>0.05
0.2	0.2	4	1.00 ± 0.08	>0.05
0.2	0.5	4	0.80 ± 0.13	>0.05
0.2	1.0	4	0.88 ± 0.05	>0.05
0.5	-	4	2.15 ± 0.18	
0.5	0.05	4	1.95 ± 0.23	>0.05
0.5	0.2	4	1.92 ± 0.18	>0.05
0.5	0.5	4	1.86 ± 0.13	>0.05
0.5	1.0	4	1.61 ± 0.09	<0.05
1.0	-	4	3.16 ± 0.21	
1.0	0.05	4	2.46 ± 0.26	>0.05
1.0	0.2	4	2.56 ± 0.19	>0.05
1.0	0.5	4	2.06 ± 0.20	<0.01
1.0	1.0	8	1.93 ± 0.15	<0.001

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

TABLE 21

Effect of L-3-Hydroxykynurenine on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	L-3-Hydroxykynurenine			
0.06	-	4	0.40 ± 0.06	
0.06	0.05	4	0.31 ± 0.02	>0.05
0.5	-	4	2.15 ± 0.18	
0.5	0.5	4	1.91 ± 0.23	>0.05
1.0	-	4	3.16 ± 0.21	
1.0	1.0	4	2.59 ± 0.33	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

TABLE 22

Effect of Anthranilic Acid on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	Anthranilic Acid			
0.06	-	4	0.40 ± 0.06	
0.06	0.05	4	0.36 ± 0.02	>0.05
0.2	-	4	0.93 ± 0.03	
0.2	0.2	4	1.08 ± 0.15	>0.05
0.5	-	4	2.15 ± 0.18	
0.5	0.5	4	2.49 ± 0.13	>0.05
1.0	-	4	3.16 ± 0.21	
1.0	1.0	4	2.91 ± 0.18	>0.05

* All incubations were 15 min in duration and were conducted in an atmosphere of 100% oxygen. The procedure is described in "Methods" Sections 7A and 7B. Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

transport of L-tryptophan are shown in Table 23. One compound appeared to stimulate tryptophan uptake: α-methyl-5-hydroxy-tryptamine. However, α-methyl-D,L-tryptophan, α-methyl-5-hydroxy-D,L-tryptophan, 4-methyl-D,L-tryptophan and 6-methyl-D,L-tryptophan had no effect on tryptophan accumulation.

We have thus attempted to delineate the structural specificity of the tryptophan transport carrier system in the light of the results shown in Tables 13-23, and we conclude as follows: the carrier mechanism exhibits an absolute requirement for the side chain aliphatic carboxyl group as well as for the α-amino group. A β-hydrogen of the aliphatic side chain however, may be substituted by a pyrrole ring, a benzene ring, a substituted benzene ring, or a branched chain aliphatic group. These conclusions are based on the observations that the only compounds which inhibited tryptophan accumulation were L-5-hydroxy-tryptophan, L-phenylalanine, L-tyrosine, L-DOPA, L-leucine, L-isoleucine, L-valine and L-kynurenine. All these compounds have an aliphatic carboxyl group and an α-amino group, but they also have different groups attached to the β-carbon. The data in Table 18 provide additional evidence concerning the structural requirements of the tryptophan carrier system. If the carboxyl group is removed (e.g. tryptamine and 5-hydroxytryptamine), or if the α-amino group is removed and the aliphatic side chain shortened by one carbon (e.g. 5-hydroxyindoleacetic

TABLE 23

Effect of Various Tryptophan Analogs on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
L-Tryptophan	Analog			
0.06	-	4	0.37 ± 0.02	
0.06	0.04 α-methyl-D,L-tryptophan	4	0.30 ± 0.04	>0.05
0.2	-	4	1.01 ± 0.10	
0.2	0.10 α-methyl-D,L-tryptophan	4	1.03 ± 0.03	>0.05
0.2	1.0 α-methyl-D,L-tryptophan	4	0.82 ± 0.11	>0.05
1.0	-	3	3.00 ± 0.10	
1.0	1.0 α-methyl-D,L-tryptophan	4	3.63 ± 0.28	>0.05
1.0	-	4	2.79 ± 0.20	
1.0	1.0 α-methyl-5-hydroxytryptamine	4	3.67 ± 0.06	<0.01
1.0	-	4	3.29 ± 0.16	
1.0	1.0 4-methyl-D,L-tryptophan		2.87 ± 0.17	>0.05
1.0	1.0 6-methyl-D,L-tryptophan	4	3.09 ± 0.16	>0.05
1.0	-	4	2.79 ± 0.20	
1.0	1.0 4-hydroxy-D,L-tryptophan	4	3.51 ± 0.24	>0.05

TABLE 23 (Cont'd)

Initial Concentration (mM)		No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
L-Tryptophan	Analog			
1.0	-	4	3.29 ± 0.16	
1.0	1.0 α-methyl-5-hydroxy-D,L-tryptophan	4	3.39 ± 0.21	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

+ Probability of difference from corresponding control.

acid and indole-3-acetic acid), or if the α-amino group is replaced by a hydroxyl group (e.g. indole-3-lactic acid), there is no effect on tryptophan accumulation. Similarly, analysis of the data detailed in Table 23 showed that substitution of the α-hydrogen by a methyl group (e.g. α-methyl-D,L-tryptophan and α-methyl-5-hydroxytryptophan) also had no effect on tryptophan transport. Thus, we postulate that the carboxyl of the aliphatic side chain and an α-amino group are required for the interaction between the tryptophan carrier and a preferred substrate. Structural requirements for the β-carbon of the aliphatic side chain however, appear to be more flexible. Thus, a pyrrole group (5-hydroxytryptophan), a benzene ring (phenylalanine), a substituted benzene ring (tyrosine, DOPA and p-chlorophenylalanine) as well as branched chain aliphatic groups (leucine, isoleucine and valine) may be substituted on the β-carbon and interaction of these compounds with the carrier mechanism will occur.

Based on these conclusions one would expect that N'formyl-L-kynurenine and L-3-hydroxykynurenine as well as L-kynurenine would interact with the tryptophan carrier system. However, only L-kynurenine inhibited tryptophan accumulation. For N'formyl-L-kynurenine, it may be that a substituent on the ortho-amino group is large enough to hinder steric interaction of this compound with the carrier. Similarly, L-3-hydroxykynurenine has an amino group and a hydroxyl group which are

ortho and meta with respect to the side chain and, again, this molecule may be too large structurally to interact with the carrier. We are unable to explain the action of α-methyl-5-hydroxytryptamine.

12. Preliminary In Vivo Experiments

A. Preamble

During the course of the *in vitro* experiments recorded in Chapters 10 and 11, we became interested in whether alterations to the physiological condition of the rat might affect the tryptophan carrier system of cerebral cortex cells. We were fortunate, in our laboratory, to have facilities available for the preparation of a synthetic diet deficient in tryptophan and for the maintainence of rats on this diet for a prolonged period. Thus, we report here on the accumulation of tryptophan by cerebral cortex slices prepared from the brain tissues of rats which had been fed a tryptophan-deficient diet for periods up to one month.

Previous investigators have observed that cortisone and hydrocortisone induce tryptophan oxygenase (Knox *et al.*, 1955; Civen *et al.*, 1959). Hydrocortisone has also been shown by Green, Joseph and Curzon (1970) to decrease the 5-hydroxytryptamine concentration of rat brain. These investigators postulate that high levels of tryptophan oxygenase activity divert tryptophan from the 5-hydroxytryptamine metabolic pathway. We questioned, however, whether these results could be attributed to an effect of hydrocortisone on the membrane carrier which transports tryptophan in brain. As these studies were in progress, a number of hypophysectomized animals were made readily

available to us. Thus, we decided to determine whether hypophysectomy might have an effect on tryptophan uptake by slices prepared from the brains of these animals.

B. Results and Discussion

(i) Dietary Deficiency

Table 24 details the results which were obtained when L-tryptophan uptake was studied in brain slices from rats which had ingested either a synthetic diet deficient in tryptophan or one which had been fortified with this amino acid. It was observed that tryptophan accumulation is not significantly altered by prolonged dietary deficiency of tryptophan.

(ii) Adrenalectomy and Hypophysectomy

The effect of adrenalectomy or hypophysectomy on the uptake of L-tryptophan by cerebral cortex slices prepared from the brains of these animals is recorded in Table 25. The accumulation of 0.06 mM, 0.2 mM and 1.0 mM medium L-tryptophan is unchanged by these operations. These preliminary results seem to indicate that there is no direct hormonal action by adrenal or pituitary hormones on the membrane carrier which transports tryptophan into cortex cells.

TABLE 24

Effect of a Tryptophan Deficient Diet on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Days on Diet	Diet	Concentration of L-Tryptophan in the Medium (mM)	No. of Detns.	Tryptophan Uptake μmoles/ml cell water	P+
3	S	1.0	4	2.94 ± 0.27	
	D	1.0	4	3.33 ± 0.04	>0.05
6	S	1.0	4	3.38 ± 0.40	
	D	1.0	4	3.27 ± 0.19	>0.05
9	S	1.0	4	3.47 ± 0.20	
	D	1.0	4	3.12 ± 0.31	>0.05
12	S	1.0	4	3.17 ± 0.30	
	D	1.0	4	3.05 ± 0.53	>0.05
15	S	1.0	3	2.43 ± 0.12	
	D	1.0	4	3.00 ± 0.22	>0.05
21	S	1.0	3	4.28 ± 0.20	
	D	1.0	4	3.13 ± 0.37	>0.05
24	S	0.2	4	1.27 ± 0.04	
	D	0.2	4	1.06 ± 0.13	>0.05
24	S	0.5	4	2.69 ± 0.22	
	D	0.5	3	2.84 ± 0.27	>0.05
24	S	1.0	4	3.53 ± 0.20	
	D	1.0	4	4.29 ± 0.10	<0.02
28	S	0.5	3	2.45 ± 0.09	
	D	0.5	4	1.97 ± 0.23	>0.05
28	S	1.0	4	3.71 ± 0.24	
	D	1.0	4	4.25 ± 0.32	>0.05

* All incubations were 15 min in duration and were conducted in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A, B and F.
"S" denotes animals on tryptophan-supplemented diet, "D" denotes animals on tryptophan deficient diet.
The supplemented diet contained 0.23% tryptophan.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

TABLE 25

Effect of Adrenalectomy and Hypophysectomy on the Accumulation of L-Tryptophan by Incubated Slices of Rat Cerebral Cortex*

Operation	Initial L-Tryptophan Concentration (mM)	No. of Detns.	Tryptophan Uptake µmoles/ml cell water	P+
---	0.06	4	0.38 ± 0.03	
Adrex	0.06	4	0.37 ± 0.03	>0.05
---	0.2	4	1.01 ± 0.07	
Adrex	0.2	4	1.08 ± 0.08	>0.05
---	1.0	8	2.75 ± 0.16	
Adrex	1.0	8	3.01 ± 0.28	>0.05
---	0.06	4	0.40 ± 0.06	
Hypox	0.06	4	0.43 ± 0.02	>0.05
---	0.2	4	1.01 ± 0.10	
Hypox	0.2	4	1.23 ± 0.05	>0.05
---	1.0	4	3.59 ± 0.24	
Hypox	1.0	4	3.46 ± 0.31	>0.05

* All incubations were 15 min in duration and were conducted at 37°C in an atmosphere of 100% oxygen.
The procedure is described in "Methods" Sections 7A and 7B. Animals were treated as described in "Materials" Section 6C.
Tabulated data represent mean values ± S.E. for L-tryptophan uptake corrected for diffusion.

\+ Probability of difference from corresponding control.

SUMMARY

1. Using radioactively labelled inulin, the size of the extracellular space of incubated rat cerebral cortex slices was found to depend upon the compounds present in the incubation medium and upon the incubation conditions. In general, however, the inulin space corrected for extracellular swelling was determined to be 22.3% of the fresh weight of tissue. The non-inulin space accounted for 59.2% of the fresh weight of tissue and the remaining 18.5% was dry weight. Tissue swelling during a 15 min incubation at 37°C was approximately 22.2% of the fresh weight of tissue.

2. Rat brain cerebral cortex slices accumulated L-tryptophan in a linear fashion during the first 20 min of incubation (the tissue:medium concentration ratio ranged between 3-4) after which the rate of accumulation fell off gradually. Maximum concentration of L-tryptophan by the slices occurred within 60 min.

3. The uptake of L-tryptophan into cerebral cortex slices is by an active mechanism. This conclusion is based upon the following observations:

   A. L-Tryptophan accumulation decreased significantly when slices were exposed to anaerobic conditions and/or low incubation temperatures.

B. The omission of Na^+ or K^+ from the incubation medium severely decreased L-tryptophan accumulation by the slices, but the absence of Ca^{2+} and/or Mg^{2+} had no effect.

C. An energy-yielding substrate such as glucose, fructose or pyruvate was required for the maximal uptake of L-tryptophan. Two Krebs cycle intermediates, citrate and succinate, were markedly less effective.

4. Kinetic analysis of the tryptophan uptake process showed that there are probably two mechanisms operating in the cortex. The first mechanism exhibits saturable Michaelis-Menten type kinetics and appears to actively concentrate L-tryptophan when concentrations of this amino acid lower than 3.0 mM are present in the incubation medium. The apparent Km of the mechanism was 0.83 mM and the apparent V max was 6.02 µmoles L-tryptophan accumulated/ml of cell water in 15 min. The second mechanism becomes evident when medium concentrations of L-tryptophan are greater than 3.0 mM and it appears to be unsaturable for medium concentrations of L-tryptophan up to at least 10 mM.

5. Monovalent cations such as Li^+, Cs^+, Rb^+, and Tl^+ had no effect on L-tryptophan uptake by the cortex slices.

6. L-tryptophan transport is inhibited by a number of amino acids notably; L-phenylalanine, D,L-p-chlorophenylalanine, L-tyrosine, L-DOPA, L-5-hydroxytryptophan, L-leucine, L-isoleucine, L-valine and L-kynurenine, but not by L-3-hydroxykynurenine, N'formyl-L-kynurenine or anthranilic acid. We concluded that L-tryptophan is transported by the carrier for the large neutral amino acids which had been detected by other investigators.

7. Additional studies with other tryptophan metabolites and some tryptophan analogs, indicated that the tryptophan carrier system of cerebral cortex slices has an absolute requirement for the carboxyl and α-amino groups on the aliphatic side chain. A β-hydrogen of the aliphatic side chain however, may be substituted by a pyrrole ring, a benzene ring, a substituted benzene ring, or by a branched chain aliphatic group.

8. Preliminary in vitro studies of slices obtained from the brains of animals which had been fed a tryptophan-deficient diet for a prolonged period indicated that this in vivo physiological alteration had no effect on tryptophan accumulation in vitro.

9. Brain slices from animals which had been subjected to adrenalectomy or hypophysectomy also showed no change in tryptophan uptake.

CLAIMS OF ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Compartmentation of the fluid associated with incubated slices of rat cerebral cortex was estimated using radioactive inulin. In general, the size of the compartments based on 100 mg fresh weight of tissue was as follows: 22.3 mg inulin space, 59.2 mg non-inulin space and 18.5 mg tissue solids. The slices swelled considerably during incubation; approximately 22.2 mg per 100 mg fresh weight of tissue during 15 min of incubation at 37°C. The inulin space value quoted here correlates well with *in vivo* estimations of the extracellular space of brain tissue. It was obtained by subtracting the amount of extracellular swelling from the experimentally determined inulin space value. We called this compartment, the "corrected" inulin space.

2. Rat brain cerebral cortex slices accumulate L-tryptophan by two processes:

   (a) an active transport mechanism which exhibits saturable Michaelis-Menten kinetics with concentrations of L-tryptophan in the medium less than 3.0 mM. This system has an apparent Km of 0.83 mM and an apparent Vmax of 6.02 µmoles L-tryptophan accumulated/ml of cell water in 15 min.

(b) Simple diffusion, a non-saturable process which can be detected when slices are incubated in media containing 3.0-10.0 mM L-tryptophan.

3. The active accumulation of 1.0 mM L-tryptophan from the medium is inhibited by anaerobiosis and/or low incubation temperatures, the absence of an energy-rich substrate such as glucose, fructose or pyruvate, medium Na^+ concentrations less than 25 mM and by complete omission of K^+. It is unaffected, however, by the absence of Ca^{2+} and/or Mg^{2+}.

4. Monovalent cations (Li^+, Cs^+, Rb^+ and Tl^+) have no effect on the uptake of 1.0 mM L-tryptophan from the medium.

5. L-Phenylalanine, D,L-p-chlorophenylalanine, L-tyrosine, L-DOPA , L-5-hydroxytryptophan, L-leucine, L-isoleucine and L-valine inhibit L-tryptophan uptake into cortex slices. Since these compounds are all large neutral amino acids, it was concluded that L-tryptophan is transported into brain cortex cells by the same carrier system that transports all the large neutral amino acids.

6. L-5-Hydroxytryptophan and L-kynurenine are the only tryptophan metabolites tested that inhibit L-tryptophan accumulation.

7. The effect of a number of tryptophan metabolites and tryptophan analogs on tryptophan accumulation into cortex slices was determined. From these studies we were able to detect some of the structural requirements of the tryptophan carrier system. The carboxyl group and the α-amino group of the aliphatic side chain are absolutely required for the interaction between the tryptophan carrier and tryptophan itself or compounds which are structurally analogous to tryptophan. A β-hydrogen, however, may be substituted by a pyrrole ring, a benzene ring, a substituted benzene ring or a branched chain alphatic group and interaction between the carrier and the compound to be transported will occur.

8. Cerebral cortex slices prepared from the brains of animals which had been fed a tryptophan-deficient diet for periods up to one month do not exhibit appreciable differences in tryptophan accumulation when compared to brain slices from animals which have received a tryptophan-supplemented diet for the same period.

9. Adrenalectomy and hypophysectomy also do not alter the uptake of L-tryptophan into slices of cerebral cortex prepared from the brains of these animals.

Abderhalden, E. (1912) Ztscher. Physiol. Chem. 77, 22.

Ames, A., Isom, J.B. and Nesbett, F.B. (1965) J. Physiol. 177, 246.

Arendt, J., Contractor, S.F. and Sandler, M. (1967) Biochem. Pharmacol. 16, 413.

Aslanyan, I.G. (1966) Dokl. Akad. Nauk. Arm. SSR 43, 215 from Guroff, G. and Lovenberg, W. The Handbook of Neurochemistry, Vol. 3 edited by Lajtha, A, Plenum Press, New York, 1970 p. 213.

Awapara, J., Sandman, R.P. and Hanly, C. (1962) Archiv. Biochem. Biophys. 98, 520.

Axelrod, J. and Weissbach, H. (1961) J. Biol. Chem. 236, 211.

Barbosa, E., Herreros, B. and Ojeda, J.L. (1971) Experientia 27, 1281.

Barbosa, E., Joanny, P. and Corriol, J. (1968) Experientia 24, 1196.

Barbosa, E., Joanny, P. and Corriol, J. (1970) C.r. Séanc. Soc. Biol. 164, 345.

Battistin, L., Grynbaum, A. and Lajtha, A. (1969) Brain Res. 16, 187.

Battistin, L., Grynbaum, A. and Lajtha A. (1971) Brain Res. 29, 85.

Belin, M.F. and Pujol, J.F. (1972a) C.R. Acad. Sc. Paris 275, 2271.

Belin, M.F. and Pujol, J.F. (1972b) C.R. Acad. Sc. Paris 275, 3017.

Belin, M.F. and Pujol, J.F. (1973) Experientia 29, 411.

Bender, D.A. and Coulson, W.F. (1972) J. Neurochem. 19, 2801.

Berg, C.P. and Potgieter, M. (1931) J. Biol. Chem. 94, 661.

Bhagvat, K., Blaschko, H. and Richter, D. (1939) Biochem. J. 33, 1338.

Birkofer, L. and Wetzel, R. (1940) Ztscher Physiol. Chem. 264, 31.

Blasberg, R. and Lajtha, A. (1965) Archiv. Biochem. Biophys. 112, 361.

Blasberg, R. and Lajtha, A. (1966) Brain Res. 1, 86.

Blaschko, H. (1952) Pharmacol. Rev. 4, 415.

Blaschko, H. and Buffoni, F. (1965) Proc. Roy. Soc. B. 163, 45.

Blaschko, H., Hagen, P. and Welch, A.D. (1955) J. Physiol. 129, 27.

Blaschko, H., Richter, D. and Schlossmann, H. (1937a) J. Physiol. 90, 1.

Blaschko, H., Richter, D. and Schlossmann, H. (1937b) Biochem. J. 31, 2187.

Boezi, J.A. and De Moss, R.D. (1961) Biochim. Biophys. Acta 49, 471.

Bonner, D.M. and Yanofsky, C. (1951) J. Nutr. 44, 603.

Bourke, R.S. and Tower, D.B. (1966) J. Neurochem. 13, 1071.

Braunshtein, A.E., Goryachenkova, E.V. and Paskhina, T. (1949) Biokhimiya 14, 163.

Bray, G.A. (1960) Analyt. Biochem. 1, 279.

Brown, R.R. and Price, J.M. (1956) J. Biol. Chem. 219, 985.

Buxton, J. and Sinclair, H.M. (1956) Biochem. J. 62, 27P.

Buzard, J.A. and Nytch, P.D. (1957) J. Biol. Chem. 229, 409.

Chirigos, M., Greengard, P. and Udenfriend, S. (1960) J. Biol. Chem. 235, 2075.

Cho-Chung, Y.S. and Pitot, H.C. (1967) J. Biol. Chem. 242, 1192.

Christensen, H.N. (1964) Proc. Natl. Acad. Sci. 51, 337.

Christenson, J.G., Dairman, W. and Udenfriend, S. (1970) Archiv. Biochem. Biophys. 141, 356.

Christenson, J.G., Dairman, W.M. and Udenfriend, S. (1971) Fed. Proc. 30, 333.

Ciusa, W. and Barbiroli, G. (1966) Boll. Soc. Ital. Biol. Sper. 42, 995 from Guroff, G. and Lovenberg, W. The Handbook of Neurochemistry Vol. 3 edited by Lajtha, A. Plenum Press, New York, 1970 p. 213.

Civen, M. and Knox, W.E. (1959) J. Biol. Chem. 234, 1787.

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

Cohen, S.R. (1972) Research Methods in Neurochemistry, Vol. 1 edited by Marks, N. and Rodnight, R. Plenum Press, New York, p. 179.

Cohen, S.R., Blasberg, R., Levi, G. and Lajtha, A. (1968) J. Neurochem. 15, 707.

Cohen, L.L. and Huang, K.C. (1964) Amer. J. Physiol. 206, 647.

Consolo, S., Garattini, S., Ghielmetti, R., Morselli, P. and Valzelli, L. (1965) Life Sc. 4, 625.

Cooper, J.R. and Melcer, I. (1961) J. Pharmacol. Exp. Therap. 132, 265.

Crane, R.K. (1965) Fed. Proc. 24, 1000.

Crnic, D.M., Hammerstad, J.P. and Cutler, R.W.P. (1973) J. Neurochem. 20, 203.

Curran, P.F., Schultz, S.G., Chez, R.A. and Fuisz, R.E. (1967) J. Gen. Physiol. 50, 1261.

Curzon, G., Friedel, J. and Knott, P.J. (1973) Nature 242, 198.

Curzon, G., Kantamaneni, B.D., Winch, J., Rojas-Bueno, A., Murray-Lyon, I.M. and Williams, R. (1973) J. Neurochem. 21, 137.

Curzon, G., Joseph, M.H. and Knott, P.J. (1972) J. Neurochem. 19, 1967.

Dalziel, K. and Dickinson, F.M. (1965) Biochem. J. 95, 311.

Danielli, J.F. (1954) Symp. Soc. Exp. Biol. 8, 502.

De Castro, F.T., Price, J.M. and Brown, R.R. (1956) J. Am. Chem. Soc. 78, 2904.

Decker, R.H., Kang, H.H., Leach, F.R. and Henderson, L.M. (1961) J. Biol. Chem. 236, 3076.

Deitrich, R.A. (1966) Biochem. Pharmacol. 15, 1911.

Dixon, M. and Webb, E.C. (1964) Enzymes, 2nd edition Longmans, London.

Duncan, R.J.S. and Sourkes, T.L. (1974) in press.

Duncan, R.J.S. and Tipton, K.F. (1971) Eur. J. Biochem. 22, 257.

Dunn, J.T. and Perkoff, G.T. (1963) Biochim. Biophys. Acta 73, 327.

Eccleston, D., Ashcroft, G.W. and Crawford, T.B.B. (1965) J. Neurochem. 12, 493.

Eccleston, D., Moir, A.T.B., Reading, H.W. and Ritchie, I.M. (1966) Br. J. Pharmac. Chemother. 28, 367.

Eddy, A.A., Mulcahy, M.F. and Thomson, P.J. (1967) Biochem. J. 103, 863.

Edlbacher, S. and Wiss, O. (1944) Helv. Chim. Acta 27, 1060.

Ellinger, A. (1906) Ber. 39, 2515 from Greenstein, J.P. and Winitz, M. Chemistry of the Amino Acids Vol. 3, John Wiley and Sons, Inc., New York (1961) p. 2321.

Ellinger, A. and Flamand, C. (1907) Ber. 40, 3029 from Greenstein, J.P. and Winitz, M. Chemistry of the Amino Acids Vol. 3, John Wiley and Sons, Inc., New York (1961) p. 2321.

Ellinger, A. and Matsuoka, Z. (1920) Ztschr. Physiol. Chem. 109, 259.

Elliott, K.A.C. (1969) from The Handbook of Neurochemistry Vol. 2 edited by Lajtha A., Plenum Press, New York, p. 103.

Erspamer, V. and Asero, B. (1952) Nature 169, 800.

Erwin, V.G. and Deitrich, R.A. (1966) J. Biol. Chem. 241, 3533.

Erwin, V.G. and Hellerman, L. (1967) J. Biol. Chem. 242, 4230.

Feldstein, A. and Williamson, O. (1968) Br. J. Pharmac. 34, 38.

Fernstrom, J.D., Larin, F. and Wurtman, R.J. (1973) Life Sciences 13, 517.

Freedland, R.A. (1963) Biochim. Biophys. Acta 73, 71.

Freedland, R.A., Wadzinski, I. M. and Waisman, H.A. (1961) Biochem. Biophys. Res. Commun. 5, 94.

Friedman, P.A., Kappelman, A.H. and Kaufman, S. (1972) J. Biol. Chem. 247, 4165.

Gal, E.M., Armstrong, J.C. and Ginsberg, B. (1966) J. Neurochem. 13, 643.

Gal, E.M., Morgan, M., Chatterjee, S.K. and Marshall, F.D. (1964) Biochem. Pharmacol. 13, 1639.

Gal, E.M. Poczik, M. and Marshall, F.D. (1963) Biochem. Biophys. Res. Commun. 12, 39.

Gholson, R.K., Nishizuka, Y., Ichiyama, A., Kawai, H., Nakamura, S. and Hayaishi, O. (1962) J. Biol. Chem. 237, PC 2043.

Gholson, R.K., Ueda, I., Ogasawara, N. and Henderson, L.M. (1964) J. Biol. Chem. 239, 1208.

Goldstein, M. and Frenkel, R. (1971) Nature 233, 179.

Goodman, F.R., Weiss, G.B. and Alderdice, M.T. (1973) Neuropharmacol. 12, 867.

Gottesfeld, Z. and Elliott, K.A.C. (1971) J. Neurochem. 18, 683.

Grahame-Smith, D.G. (1964a) Biochem. J. 92, 52P.

Grahame-Smith, D.G. (1964b) Biochim. Biophys. Acta 86, 176.

Grahame-Smith, D.G. (1971) J. Neurochem. 18, 1053.

Grahame-Smith, D.G., Parfitt, A., Warnock, M. and Taylor, M. (1969) Proc. 2nd Int. Meet. Int. Soc. Neurochem. Milan, p. 199.

Grahame-Smith, D.G. and Parfitt, A.G. (1970) J. Neurochem. 17, 1339.

Green, A.R. and Curzon, G. (1970) Biochem. Pharmacol. 19, 2061.

Green, A.R., Joseph, M.H. and Curzon, G. (1970) The Lancet 1, 1288.

Groth, U. and Rosenberg, L.E. (1972) J. Clin. Invest. 51, 2130.

Guroff, G. (1972) from Basic Neurochemistry edited by Albers, R.W., Siegel, G.J., Katzman, R. and Agranoff, B.W. Little Brown and Company, Boston, p. 191.

Guroff, G., King, W. and Udenfriend, [redacted] (1961) J. Biol. Chem. 236, 1773.

Guroff, G. and Udenfriend, S. (1962) J. Biol. Chem. 237, 803.

Guroff, G. and Udenfriend, S. (1964) from Progress In Brain Research, The Developing Rat Brain Vol. 9 edited by Himwich, W.A. and Himwich, H.E., Elsevier, Amsterdam, p. 187.

Haavaldsen, R. (1962) Nature 196, 577.

Harada, M. and Nagatsu, T. (1969) Experientia 25, 583.

Hare, M.L.C. (1928) Biochem. J. 22, 968.

Hawkins, J. (1952) Biochem. J. 50, 577.

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

Hayaishi, O. and Stanier, R.Y. (1951) J. Bacteriol. 62, 691.

Henderson, L.M. (1949) J. Biol. Chem. 178, 1005.

Henderson, L.M. and Hirsch, H.M. (1949) J. Biol. Chem. 181, 667.

Higuchi, K. and Hayaishi, O. (1967) Archiv. Biochem. Biophys. 120, 397.

Hirata, F., Tokuyama, T., Senoh, S. and Hayaishi, O. (1973) Proc. 4th Int. Meet. Int. Soc. Neurochem. Tokoyo, p. 391.

Hopkins, F.G. and Cole, S.W. (1901) J. Physiol. 27, 418.

Hosoda, S. and Glick, D. (1966) J. Biol. Chem. 241, 192.

Ichiyama, A., Nakamura, S., Kawai, H., Honjo, T., Nishizuka, Y., Hayaishi, O. and Senoh, S. (1965) J. Biol. Chem. 240, 740.

Jacquez, J.A. (1961) Amer. J. Physiol. 200, 1063.

Jakoby, W. (1954) J. Biol. Chem. 207, 657.

Jakoby, W.B. and Bonner, D.M. (1953) J. Biol. Chem. 205, 699.

Jakoby, W.B. and Bonner, D.M. (1956) J. Biol. Chem. 221, 689.

Jequier, E., Lovenberg, W. and Sjoerdsma, A. (1967) Mol. Pharmacol. 3, 274.

Jequier, E., Robinson, D.S., Lovenberg, W. and Sjoerdsma, A. (1969) Biochem. Pharmacol. 18, 1071.

Joanny, P., Barbosa, E. and Corriol, J. (1968) J. Physiologie Supp. 60, 265.

Joanny, P., Corriol, J. and Hillman, H. (1969) Proc. 2nd Int. Meet. Int. Soc. Neurochem. Milan, p. 229.

Joanny, P., Natali, J.P., Hillman, H. and Corriol, J. (1973) Biochem. J. 136, 77.

Kaihara, M. and Price, J.M. (1962) J. Biol. Chem. 237, 1727.

Karobath, M., Diaz, J.L. and Huttunen, M.O. (1971) Europ. J. Pharmacol. 14, 393.

Kearney, E.B., Salach, J.I., Walker, W.H., Seng, R. and Singer, T.P. (1971) Biochem. Biophys. Res. Commun. 42, 490.

Knapp, S. and Mandell, A.J. (1973) Science 180, 645.

Knott, P.J. and Curzon, G. (1972) Nature 239, 452.

Knox, W.E. and Auerbach, V.H. (1955) J. Biol. Chem. 214, 307.

Knox, W.E. and Mehler, A.H. (1950) J. Biol. Chem. 187, 419.

Kohn, H.I. (1937) Biochem. J. 31, 1693.

Kotake, Y. and Ito, N. (1937) J. Biochem. 25, 71.

Kotake, Y. and Masayama, T. (1936) Ztscher. Physiol. Chem. 243, 237.

Krebs, H.A. (1935) Biochem. J. 29, 1620.

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

Kveder, S., Iskrić, S., and Keglević, D. (1962) Biochem. J. 85, 447.

Laduron, P. and Belpaire, F. (1968) Biochem. Pharmacol. 17, 1127.

Lahiri, S. and Lajtha, A. (1964) J. Neurochem. 11, 77.

Lancaster, G.A. and Sourkes, T.L. (1972) Cdn. J. Biochem. 50, 791.

Latt, N., Rippey, J.J. and Stacey, R.S. (1968) Brit. J. Pharmacol. 32, 427P.

Lerner, A.B., Case, J.B. and Takahashi, Y. (1960) J. Biol. Chem. 235, 1992.

Levi, G., Blasberg, R. and Lajtha, A. (1966) Archiv. Biochem. Biophys. 114, 339.

Lin, E.C.C., Hagihira, H. and Wilson, T.H. (1962) Amer. J. Physiol. 202, 919.

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

Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658.

Lipsett, D., Madras, B.K., Wurtman, R.J. and Munro, H.N. (1973) Life Sci. 12, 57.

Liu, Y.P., Ambani, L.M. and Van Woert, M.H. (1972) J. Neurochem. 19, 2237.

Loh, H.H. and Berg, C.P. (1971) J. Nutrition 101, 465.

Loh, H.H. and Berg, C.P. (1972) J. Nutrition 102, 1331.

Longenecker, J.B. and Snell, E.E. (1955) J. Biol. Chem. 213,229.

Lovenberg, W., Jequier, E. and Sjoerdsma, A. (1967) Science 155, 217.

Lovenberg, W., Levine, R.J. and Sjoerdsma, A. (1965) Biochem. Pharmacol. 14, 887.

Lovenberg, W., Weissbach, H. and Udenfriend, S. (1962) J. Biol. Chem. 237, 89.

McGeer, P.L., Bagchi, S.P. and McGeer, E.D. (1965) Life Sci. 4, 1859.

McIssac, W.M. and Page, I.H. (1959) J. Biol. Chem. 234, 858.

McMenamy, R.H. and Oncley, J.L. (1958) J. Biol. Chem. 233, 1436.

Madras, B.K., Cohen, E.L., Fernstrom, J.D., Larin, F., Munro, H.N. and Wurtman, R.J. (1973) Nature 244, 34.

Mason, M. (1954) J. Biol. Chem. 211, 839.

Matsuoka, Z. and Lakemura, S. (1922) J. Biochem. 1, 175.

Mehler, A.H. (1956) J. Biol. Chem. 218, 241.

Mehler, A.H. and Knox, W.E. (1950) J. Biol. Chem. 187, 431.

Mehler, A.H. and May, E.L. (1956) J. Biol. Chem. 223, 449.

Meister, A. (1965) Biochemistry of the Amino Acids Vol. II, Academic Press, New York, p. 841.

Mandel, L.B. (1914-15) The Harvey Lectures 10, 101.

Miller, I.L., Tsuchida, M. and Adelberg, E.A. (1953) J. Biol. Chem. 203, 205.

Mitchell, R.A., Kang, H.H. and Henderson, L.M. (1963) J. Biol. Chem. 238, 1151.

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

Moir, A.T.B. and Eccleston, D. (1968) J. Neurochem. 15, 1093.

Munck, B.G. (1966) Biochim. Biophys. Acta 126, 299.

Nakamura, S., Ichiyama, A. and Hayaishi, O. (1965) Fed. Proc. 24, 604.

Nakamura, S., Ikeda, M., Tsuji, H., Nishizuka, Y. and Hayaishi, O. (1963) Biochem. Biophys. Res. Commun. 13, 285.

Neame, K.D. (1964) J. Neurochem. 11, 67.

Neame, K.D. (1966) J. Physiol. 185, 627.

Neame, K.D. (1968) in Progress in Brain Research, Brain Barrier Systems Vol. 29 edited by Lajtha, A. and Ford, D.H., Elsevier, Amsterdam, p. 185.

Nishizuka, Y. and Hayaishi, O. (1963a) J. Biol. Chem. 238, PC483.

Nishizuka, Y. and Hayaishi, O. (1963b) J. Biol. Chem. 238, 3369.

Oates, J.A. and Sjoerdsma, A. (1961) Proc. Soc. Exp. Biol. Med. 108, 264.

Ogasawara, N., Gander, J.E. and Henderson, L.M. (1966) J. Biol. Chem. 241, 613.

Ogasawara, N., Hagino, Y. and Kotake, Y. (1962) J. Biochem. 52, 162.

Oxender, D.L. and Christensen, H.N. (1963) J. Biol. Chem. 238, 3686.

Packman, P.M. and Jakoby, W.B. (1965) J. Biol. Chem. 240, PC4107.

Pappius, H.M., (1969) from The Handbook of Neurochemistry Vol. 2 edited by Lajtha, A., Plenum Press, New York, p. 1.

Pappius, H.M. and Elliott, K.A.C. (1956) Can. J. Biochem. and Physiol. 34, 1007.

Pappius, H.M., Klatzo, I. and Elliott, K.A.C. (1962) Can. J. Biochem. and Physiol. 40, 885.

Pappius, H.M., Rosenfeld, M., Johnson, D.M. and Elliott, K.A.C. (1958) Can. J. Biochem. and Physiol. 36, 217.

Peng, Y., Gubin, J., Harper, A.E., Vavich, M.G. and Kemmerer, A.R. (1973) J. Nutr. 103, 608.

Perez-Cruet, J. and Eichelman, B. (1972) Fed. Proc. 31, 579.

Potashner, S.J. and Johnstone, R.M. (1971) Biochim. Biophys. Acta 233, 91.

Pugh, C.E.M. and Quastel, J.H. (1937a) Biochem. J. 31, 286.

Pugh, C.E.M. and Quastel, J.H. (1937b) Biochem. J. 31, 2306.

Rapport, M.M., Green, A.A. and Page, I.H. (1948) Science 108, 329.

Raskin, N.H. and Sokoloff, L. (1968) Science 162, 131.

Raskin, N.H. and Sokoloff, L. (1972) J. Neurochem. 19, 273.

Reiser, S. and Christiansen, P.A. (1973) Life Sciences 12, 25.

Renson, J., Weissbach, H. and Udenfriend, S. (1962) J. Biol. Chem. 237, 2261.

Riggs, T.R., Coyne, B.A. and Christensen, H.N. (1954) J. Biol. Chem. 209, 395.

Robinson, D., Lovenberg, W. and Sjoerdsma, A. (1968) Archiv. Biochem. Biophys. 123, 419.

Rodriguez De Lores Arnaiz, G. and De Robertis, E. (1964) J. Neurochem. 11, 213.

Rogers, Q.R. and Harper, A.E., (1965) J. Nutrition 87, 267.

Rose, W.C. (1938) Physiol. Rev. 18, 109.

Rosengren, E. (1960) Acta Physiol. Scand. 49, 364.

Saito, Y., Hayaishi, O. and Rothberg, S. (1957) J. Biol. Chem. 229, 921.

Sandler, M., Spector, R.G., Ruthven, C.R.J. and Davison, A.N. (1960) Biochem. J. 74, 42P.

Sato, T.L., Jequier, E., Lovenberg, W. and Sjoerdsma, A. (1967) Europ. J. Pharmacol. 1, 18.

Schanberg, S.M. (1963) J. Pharm. Exptl. Therap. 139, 191.

Schanberg, S. and Giarman, N.J. (1960) Biochim. Biophys. Acta 41, 556.

Schayer, R.W. (1950) J. Biol. Chem. 187, 777.

Schubert, J. (1973) Psychopharmacologia 32, 301.

Schurr, P.E., Thompson, H.T., Henderson, L.M., Williams, J.N. and Elvehjem, C.A. (1950) J. Biol. Chem. 182, 39.

Sims, K.L., Davis, G.A. and Bloom, F.E. (1973) J. Neurochem. 20, 449.

Sjoerdsma, A., Weissbach, H. and Udenfriend, S. (1956) Am. J. Med. 20, 520.

Smith, S.E. (1963) Brit. J. Pharmacol. 20, 178.

Sourkes, T.L., Missala, K. and Oravec, M. (1970) J. Neurochem. 17, 111.

Sourkes, T.L. and Townsend, E. (1955) Can. J. Biochem. and Physiol. 33, 735.

Spencer, R.P., Bow, T.M. and Markulis, M.A. (1962) Amer. J. Physiol. 202, 171.

Spencer, R.P. and Samiy, A.H. (1957) Amer. J. Physiol. 199, 1033.

Stevens, C.O. and Henderson, L.M. (1959a) J. Biol. Chem. 234, 1188.

Stevens, C.O. and Henderson, L.M. (1959b) J. Biol. Chem. 234, 1191.

Sund, H. and Theorell, H. (1963) from The Enzymes Vol. 7 edited by Boyer, P.D., Lardy, H. and Myrbäck, K. Academic Press, New York, p. 25.

Tabakoff, B., Anderson, R. and Alivisatos, S.G.A. (1973) Mol. Pharmacol. 9, 428.

Tabakoff, B. and Erwin, V.G. (1970) J. Biol. Chem. 245, 3263.

Tabor, C.W., Tabor, H. and Rosenthal, S. (1954) J. Biol. Chem. 208, 645.

Tagliamonte, A., Biggio, G. and Gessa, G.L. (1971) Riv. di Farmacol. e Terapia 11, 251.

Tagliamonte, A., Biggio, G., Vargiu, L. and Gessa, G.L. (1973) Life Sci. 12, 277.

Tagliamonte, A., Tagliamonte, P., Perez-Cruet, J., Stein, S. and Gessa, G.L. (1971) J. Pharmacol. and Exptl. Therap. 177, 475.

Takahashi, H., Kaihara, M. and Price, J.M. (1956) J. Biol. Chem. 223, 705.

Tanaka, T. and Knox, W.E. (1959) J. Biol. Chem. 234, 1162.

Tangen, O., Fonnum, F. and Haavaldsen, R. (1965) Biochim. Biophys. Acta 96, 82.

Tipton, K.F. (1968) Biochim. Biophys. Acta 159, 451.

Tsuda, H., Noguchi, T. and Kido, R. (1972) J. Neurochem. 19, 887.

Tyce, G.M., Flock, E.V. and Owen, C.A. (1964) from Progress in Brain Research, The Developing Rat Brain Vol. 9, edited by Himwich, W.A. and Himwich, H.E., Elsevier, Amsterdam, p. 198.

Tyce, G.M., Muenter, M.D. and Owen, C.A. (1970) Mayo Clin. Proc. 45, 438.

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

Udenfriend, S., Titus, E., Weissbach, H. and Peterson, R.E. (1956) J. Biol. Chem. 219, 335.

Ueno, Y., Hayaishi, K. and Shukuya, R. (1963) J. Biochem. 54, 75.

Ussing, H.H. (1947) Nature 160, 262.

Ussing, H.H. (1949) Physiol. Rev. 29, 127.

Vahvelainen, M.L. and Oja, S.S. (1972) Brain Res. 40, 477.

Varon, S. and McIlwain, H. (1961) J. Neurochem. 8, 262.

Vescia, A. and Di Prisco, G. (1962) J. Biol. Chem. 237, 2318.

Vidaver, G.A. (1964a) Biochemistry 3, 662.

Vidaver, G.A. (1964b) Biochemistry 3, 795.

Vidaver, G.A. and Shepherd, S.L. (1968) J. Biol. Chem. 243, 6140.

Weber, L.J. and Horita, A. (1965) Biochem. Pharmacol. 14, 1141.

Weissbach, H., Bogdanski, D.F., Redfield, B.G. and Udenfriend, S. (1957) J. Biol. Chem. 227, 617.

Weissbach, H., Redfield, B.G. and Axelrod, J. (1960) Biochim. Biophys. Acta 43, 352.

Weissbach, H., Redfield, B.G. and Udenfriend, S. (1957) J. Biol. Chem. 229, 953.

Werle, E. and Aures, D. (1959) Ztscher. Physiol. Chem. 316, 45.

Werle, E. and Mennicken, G. (1937) Biochem. Ztscher. 291, 325.

Willcock, E.G. and Hopkins, F.G. (1906) J. Physiol. 35, 88.

Wiseman, G. (1956) J. Physiol. 133, 626.

Wiss, O. (1953) Ztscher. Physiol. Chem. 293, 106.

Wiss, O. and Bettendorf, G. (1957) Ztscher. Physiol. Chem. 306, 145.

Wiss, O. and Fuchs, H. (1950) Experientia 6, 472.

Wiss, O. and Weber, F. (1956) Ztscher. Physiol. Chem. 304, 232.

Yamada, H. and Yasunobu, K.T. (1962) J. Biol. Chem. 237, 3077.

Yamada, H. and Yasunobu, K.T. (1963) J. Biol. Chem. 238, 2669.

Yamamoto, S. and Hayaishi, O. (1967) J. Biol. Chem. 242, 5260.

Yanofsky, C. and Bonner, D.M. (1950) Proc. Natl. Acad. Sci. 36, 167.

Yoshida, H.,Kaniike, K. and Namba, J. (1963b) Nature 198, 191.

Yoshida, H., Namba, J.,Kaniike, K. and Imaizumi, R. (1963a) Jap. J. Pharmacol. 13, 1.

Youdim, M.B.H. and Sourkes, T.L. (1972) Adv. Biochem. Psychopharmac. 5, 45.

Yuwiler, A. (1973) J. Neurochem. 20, 1099.

Yuwiler, A., Geller, E. and Eidusan, S. (1959) Archiv. Biochem. Biophys. 80, 162.

ADDENDUM 1

Further evidence from the laboratory of Hirata, Hayaishi, Tokuyama and Senoh (1974)* indicates that the partially purified rabbit brain enzyme (tryptophan 2,3-dioxygenase) is capable of cleaving the pyrrole moiety of melatonin to yield N^{γ}-acetyl-N^{2}-formyl-5-methoxykynuranamine. This compound can then be degraded to N^{γ}-acetyl-5-methoxy-kynurenamine by the formamidase enzyme. Hirata *et al.* (1974) have verified these results *in vivo*.

* Hirata, F., Hayaishi, O., Tokuyama, T.; and Senoh, S. (1974) J. Biol. Chem. 249, 1311.