RELATIONSHIPS BETWEEN TRYPTOPHAN, DIET, 5-HYDROXYTRYPTAMINE METABOLISM, 5-HYDROXYTRYPTAMINE FUNCTION AND BEHAVIOR

by

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To my daughter

Kiri-Marie

whose life has been affected

by this work

as much as my own

ABSTRACT

The role of tryptophan availability in the regulation of 5-hydroxytryptamine (5HT) metabolism and function has been examined in rats and humans, by manipulation of dietary components. It was demonstrated that in the rat, protein and carbohydrate can alter 5HT levels in peripheral tissues as well as brain. A new method for the determination of 5HT in the cisternal cerebrospinal fluid (CSF) of the rat was developed and used as an index of functional 5HT, using pharmacological agents known to act on 5HT function. Using this technique, it was then shown that tryptophan administration potentiates the release of 5HT when the animals are aroused, and thus when their 5HT neurons are firing at high rates. However, the smaller changes in brain 5HT after ingestion of protein or carbohydrate did not lead to altered CSF 5HT. In humans, similar conclusions were drawn. The administration of an amino acid mixture deficient in tryptophan significantly altered macronutrient selection, a behavior thought to be mediated by lowered tryptophan availability and 5HT function. However, when protein or carbohydrate breakfasts were given, no effect on macronutrient selection was observed, suggesting that the physiological changes resulting from dietary intake were not affecting functional 5HT. The final study measured amine precursors and metabolites in human lumbar CSF after the administration of protein or carbohydrate breakfasts and supported this hypothesis. No effects on CSF tryptophan or 5-hydroxyindoleacetic acid, the major metabolite of 5HT, were observed. In conclusion, the results of these studies suggest that, though large changes in precursor availability can alter 5HT function, the effects of dietary intake on 5HT metabolism are not normally of sufficient magnitude to alter 5HT function in rats or humans, or brain 5HT metabolism in humans.

RÉSUMÉ

En manipulant certains composants de la diète, le rôle de la disponibilité du tryptophane dans le métabolisme et la fonction de la 5-hydroxytryptamine (5HT) fut examiné chez le rat et l'homme. It á eté démontré chez le rat que les proteines et les glucides altèrent les niveaux de la 5HT dans les tissues periphériques et également dans le cerveau.

En utilisant des agents pharmacologiques reconnus comme agissant sur la fonction de la 5HT, une nouvelle méthode pour sa détermination dans le liquide céphalorachidien (LCR) cisterne fut validé comme index du 5HT fonctionne. D'apres cette technique it a été montré que l'administration de tryptophane (TRP) chez le rat accentue les effets de la 5HT quand ces animaux sont en éveil, et donc quand les neurones serotoniques fonctionnent a grand vitesse. Cependant, les légers changements dans le niveau de la 5HT du cerveau après l'ingestion de proteine ou de glucide n'entrainèrent pas l'altération de la 5HT.

Chez l'homme, des conclusions similaires LCR furent tirées. L'administration d'un mélange d'acides aminés depourvu de TRP, changea d'une facon significative la sélection de protéine, ceci étant probablement médié par une baisse de la disponibilité du TRP et de la fonction de la 5HT. Cependant, quand des dejeuners de protéines ou de glucide furent donnés, aucun effet sur la sélection de ces élements n'a été observé suggérant que les changements physiologiques causés par la diète n'avaient pas d'effets sur le 5HT fonctionnel. L'étude final qui comportait la determination des précurseurs aminés et des métabolites dans le LCR lombaire chez l'homme après l'administration de déjeûners de proteine ou de glucide a supporté cette hypothèse. Aucun effet sur le tryptophane ou l'acide 5-hydroxyindoleacetique, le métabolite majeur de

la 5HT, ne fut observé.

En conclusion, les résultats de ces études suggèrent que même si de grands changements dans la disponibilité du precurseur peuvent altérer la fonction de la 5HT, les effets de la diète sont normalement insuffisant pour altérer la fonction de la 5HT chez le rat et l'homme ou son métabolisme dans le cerveau humain.

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PREFACE

"The Candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. The thesis conforms to other requirements explained in the Guidelines concerning Thesis Preparation, McGill University, Faculty of Graduate Studies and Research"

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Chapter 2: Teff, K.L. and Young, S.N. 1988. Effects of carbohydrate and protein administration on rat tryptophan and 5-hydroxytryptamine:differential effects on the brain, intestine, pineal and pancreas. Can. J. Physiol. Pharm. In Press.

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Chapter 4: Teff, K.L. and Young, S.N. 1988. The effect of tryptophan, protein and carbohydrate administration on 5-hydroxytryptamine synthesis and release in the rat. Manuscript in preparation.

Chapter 5: Young, S.N., Tourjman, S.V., Teff, K.L. , Pihl, R.O. and Anderson, G.H. 1988. The effect of lowering plasma tryptophan on food selection in normal males. Pharm. Biochem. Behav. In Press.

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Chapter three: Dr. G.M. Anderson developed the method for the measurement of 5-hydroxytryptamine in rat cerebrospinal fluid and in this particular experiment carried out the determinations. The Candidate was resposible for the conception, planning, performing and analyzing of the experiment as well as the writing of the final journal article.

Chapter five: the Candidate was not involved in the original conception of the experiment but later at the time of the experiment helped in the design, and organized and carried out the work as well as analyzed the data and wrote the paper. Dr. Tourjman, a medical student, helped to run the experiment, supervised the subjects and took blood samples. Dr. Pihl advised on various aspects of design and data analysis, while Dr. Anderson carried out the measurement of the amino acids other than tryptophan in the blood samples.

Chapter six: Dr. J. Blundell supplied the food questionnaires

Chapter seven: Dr. L. Marchand and Dr. M. Botez were responsible for the clinical aspects of the study and performed the lumbar punctures.

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LIST OF ABBREVIATIONS

AMP	adenosine monophosphate
BBB	blood-brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
EC	enterochromaffin cells
ECF	extracellular fluid
F	fluorometric
HPLC	high performance liquid chromatography
g	gram
5HIAA	5-hydroxyindoleacetic acid
5HT	5-hydroxytryptamine
5HTP	5-hydroxytryptophan
I.G.	intragastric
I.P.	intraperitoneal
Km	Michaelis-Menten constant
LC	liquid chromatography
M	molar
mM	millimolar
MAO	monoamine oxidase
MAOI	monoamine oxidae inhibitor
mg	milligram
ml	milliliter
NAD	nicotinamide adenine dinucleotide
NMS	N-methylserotonin
ng	nanogram
pg	picogram
SD	standard deviation

LIST OF ABBREVIATIONS

SE	standard error
S/N	signal to noise ratio
TRP	tryptophan
ug	microgram
ul	microliter
umol	micromole
Vmax	maximum velocity

1.1.1 Introduction

Conceptions and misconceptions related to the behavioral effects of food have probably existed since the beginning of man. Aggression, mood, sleep and sex are amoung the most common behaviors which seem to be associated with food mythologies. Specific foods are often attributed with certain behavioral properties. These foods differ from country to country as many of the beliefs are culture-bound. Thus, depending on whether one is Nigerian, North American or Indian, one could consume either eggs, oysters or onions to increase sexual activity, according to local custom. Though one can only speculate whether the ideas originated from personal experience or religious taboos, the very existence of these popular myths suggests the widespread acceptance that diet is capable of altering behavior.

If diet or dietary components are capable of altering behavior, then one must assume that the mediation of this behavioral change occurs in the central nervous system and that the mediator is some type of neurotransmitter or modulator. Therefore, a logical sequence of events is established. Dietary intake alters brain metabolism which subsequently brings about a change in behavior. If the change in metabolism alters behavior, then this implies a change in the function of the neurochemical component. The experimental work in this thesis is concerned with the effects of the dietary macronutrients, protein and carbohydrate and how they alter the metabolism and function of one particular neurotransmitter, 5-hydroxytryptamine (5HT). As will be discussed in chapter 1, 5HT is synthesized from an essential amino acid tryptophan and thus is the central component in the diet, brain and behavior relationship.

1.2.1 Tryptophan, the amino acid precursor to 5-hydroxytryptamine

1.2.2 Dietary intake and peripheral metabolism of tryptophan

In mammalian protein metabolism, 8 out of 20 amino acids normally found in protein are considered essential inasmuch as they cannot be synthesized in adequate amounts and therefore must be obtained by dietary intake. To promote growth in the young and maintain nitrogen equilibrium in the adult, animals must ingest protein that contains adequate amounts of the essential amino acids. Tryptophan has the lowest dietary requirement of any of the essential amino acids. For a 100g rat, the daily requirement is 0.01 g (Munro, 1970) but the dietary intake is substantially higher, as it is in the range of 0.03-0.04g. A similar situation exists in humans; the suggested minimum requirement is 0.16 and 0.25g, for women and men respectively (Rose, 1957) while 1-1.5g are consumed daily in the form of protein (Murphy et al., 1974).

Tryptophan and other amino acids are liberated from digested protein by enzymes in the stomach and intestine. The free amino acids as well as di and tripeptides are transported across the intestinal mucosal barrier. The process of amino acid transport is a Na+ dependent, active system (Steven et al., 1984) which occurs against a concentration gradient (Cohen and Huang, 1964) and can be inhibited with high concentrations of the substrate (Spencer and Samiy, 1960). Both phenylalanine and tyrosine can compete with tryptophan for transport (Cohen and Huang, 1964). In addition, there is evidence that intestinal amino acid transport systems can increase their rate of transport with a high protein diet (Wolffram and Scharrer, 1984).

Once absorbed, tryptophan is carried to the liver in the hepatic

portal blood. Initially after protein ingestion, amino acid concentrations will be higher in portal blood than in venous, the loss being due to uptake in the tissues, primarily the liver, for protein synthesis (Christensen, 1964). Quantitatively, protein synthesis is the most important pathway and the only reversible one for tryptophan. Though skeletal muscle accounts for the largest reservoir of amino acids in the body, the liver is the major site of labile protein deposition. It appears that tryptophan plays a unique regulatory role in protein synthesis (Munro, 1970). This is probably because it is the amino acid present in the lowest concentration in the free amino acid pools of the individual tissues; it is also the least abundant amino acid in the protein being synthesized. Therefore, tryptophan can become the rate-limiting amino acid in the protein synthetic machinery. A tryptophan deficient amino-acid mixture can effect liver RNA metabolism by altering the polysomal population (Munro, 1968). Addition of tryptophan can increase ribosome aggregation and hepatic protein synthesis (Sidransky, 1971) thereby accelerating the incorporation of tryptophan into new protein. In rats, acute depletion of tryptophan by the administration of a tryptophan-deficient amino acid diet will not inhibit protein synthesis competely but will result in a rapid lowering of serum and tissue content of the amino acid (Biggio et al., 1974). As already stated, protein stores in the liver are labile and there is a constant flux between protein synthesis and catabolism during the absorbtive and postabsorbtive phases of digestion.

The most important route of tryptophan catabolism is the kynurenine pathway, which can ultimately lead either to the formation of nicotinamide adenine dinucleotide (NAD) or the complete catabolism of

tryptophan to carbon dioxide. Only a small proportion of tryptophan entering this pathway is synthesized into NAD, as tryptophan is also the precursor of other metabolites which are formed along the pathway. The first and rate-limiting enzyme (Young et al., 1974b) is tryptophan pyrrolase (L-tryptophan-2,3-dioxygenase, EC 1.13.1.12) which is found mostly in the liver. The enzyme can be induced by glucocorticoids and tryptophan (Knox,1966). Administration of a tryptophan load can increase the enzyme concentration, thereby increasing the rate of tryptophan breakdown to carbon dioxide (Young and Sourkes, 1975). On the other hand, it can also influence oxidation without altering enzyme protein levels due to increased saturation of the enzyme with its heme cofactor and with tryptophan itself. Whether the enzyme is induced or not depends on the tryptophan content of the liver which in turn influences the degree of enzyme saturation. Ultimately, high levels of plasma tryptophan will be rapidly metabolized by tryptophan pyrrolase. Mammalian tissue also contains indoleamine pyrrolase (indole-2, 3-dioxygenase) which catalyses the same reaction but which has broader specificity. It is not known what proportion of tryptophan degradation is catalyzed by each enzyme, but the proportion probably varies from species to species.

Induction of tryptophan pyrrolase without precursor loading by glucocorticoids can lower tissue levels of tryptophan, including in the brain (Green and Curzon, 1968; Green et al.,1975; Green and Curzon, 1975). Hydrocortisone given to rats will increase tryptophan catabolism by pyrrolase threefold (Green and Curzon, 1968). Free tryptophan stores in the blood, muscle, liver and brain decline. This reduction can be accounted for by the increased rate of tryptophan catabolism (Young,

1981). The reduction in free tryptophan will initiate a compensatory increase in protein catabolism which will limit the extent of the trypophan decline. A similar pattern of a decline in tissue and blood tryptophan (Sourkes, 1971) with an increase in protein catabolism has also been seen after the administration of alpha-methyltryptophan (Oravec and Sourkes, 1969; Oravec and Sourkes, 1970), a substrate analog of tryptophan which causes a long lasting induction of tryptophan pyrrolase. Thus, tryptophan pyrrolase plays an important role in the regulation of plasma tryptophan levels and as discussed below can influence the level of this amino acid.

1.2.3 Tryptophan transport into the central nervous system

Entry of tryptophan into brain cells involves transport across two separate barriers. Tryptophan in the plasma must first be transported across the blood-brain barrier (BBB) into the extracellular fluid and then from the extracellular fluid across the cell membrane. Although the majority of studies have been on brain tissue, transport across the blood-brain barrier is probably of greater physiological importance in the regulation of brain amino acid levels. This is because of the high affinity, low capacity carrier at the blood-brain barrier. In contrast, at the brain cell membrane the capacity of the carrier is much greater and unlikely to be saturated (Pardridge and Oldendorf, 1975). Despite differences in the Km and Vmax, similarities in substrate specificity and competitive inhibition exist in the two membrane systems (Lajtha, 1974; Young and Sourkes, 1977).

The blood-brain barrier is located within the brain endothelial cells which form a continuous barrier between blood and brain interstitial

fluid. Amino acids are transported across the barrier by saturable, class specific carriers. Distinct transport systems exist for acidic, basic and neutral amino acids (Oldendorf, 1971; Oldendorf and Szabo, 1976). Unlike other cell transport systems where two carriers have been defined for the neutral amino acids (Christensen, 1969), only one such system seems to be operative at the blood brain barrier (Wade and Katzman, 1978). This corresponds to the L-system, which is sodium independent and regulates bidirectional amino acid movement across cell membranes (Christensen, 1969).

An additional difference in transport at the blood brain barrier with respect to other membrane systems is the low Km, i.e. high affinity of the carrier. The Km of the carrier for tryptophan alone is approximately 0.1mM, while total plasma tryptophan ranges from 0.04 to 0.1mM. Therefore, under normal physological conditions, the carrier is unsaturated and increases in plasma levels can increase uptake. But tryptophan shares this transport system and must compete with other neutral amino acids including phenylalanine, tyrosine, leucine, isoleucine, valine, histidine and methionine. The competition between tryptophan and the other large neutral amino acids lowers the affinity of the carrier resulting in an apparent Km for tryptophan of 0.4 to 0.6mM. Competition between amino acids can take place when the Km of the carrier is of the same order of magnitude as the concentrations of the amino acids in the plasma (Oldendorf, 1971; Pardridge, 1977). Because of this, brain levels of the neutral amino acids do not simply follow alterations in plasma levels. It has been postulated that the ratio of tryptophan to the sum of the competing amino acids is an index of tryptophan availability to the brain (Fernstrom and Wurtman, 1972; Ashley and

Anderson, 1975). The role of the tryptophan ratio will be discussed in greater detail in the following section, as dietary components exert differential effects on the neutral amino acids. In other tissues, such as the liver, kidney and intestine, Km's are much higher than plasma levels and no competition occurs (Pardridge and Oldendorf, 1977). In the pancreas, there is some evidence that competition does take place (see chapter two).

Measurement of amino acid uptake at the blood brain barrier has been studied primarily using the "Oldendorf technique" (Oldendorf, 1971). This involves the injection of a bolus solution containing ^{14}C-labeled tryptophan and ^{3}H-water under sufficient pressure to temporarily stop blood flow. The bolus is allowed to pass through the brain for 15s by which time it is washed out of the circulation, then the rat is decapitated. The ratio of ^{14}C to ^{3}H in the bolus is compared to the ratio found in the brain, resulting in the brain uptake index (BUI), which is expressed as a percentage. The BUI for L-tryptophan was originally found to be 33% (Oldendorf, 1971) but varies inversely with increasing concentrations (Pardridge and Oldendorf, 1975). The BUI for D-tryptophan is 3.5%, only slightly above blank levels, suggesting that uptake is primarily stereospecific (Yuwiler, 1973). Other in vivo methods, such as in situ brain perfusion (Smith et al., 1987) and intravenous injection of a radio-labeled amino acid under steady state conditions (Banos et al., 1973) have also been used to measure brain tryptophan influx. There is a fairly good correspondence between these two techniques. All three methods show that uptake occurs both by saturable, active transport and non-saturable diffusion, though the second component is small. As well, higher rates of influx for essential

amino acids as compared to non-essential have been demonstrated. The rapid rate of exchange of amino acids from blood to brain (Lajtha, 1959) and evidence for the transport of amino acids from the brain despite elevated plasma levels (Lajtha and Toth, 1961) suggests that efflux can significanly effect brain amino acid levels (Young and Sourkes, 1977) but this process has not been studied to any extent.

Having been transported into the brain extracellular fluid, tryptophan must then be transported across brain cell membrane. Brain tissue slices, synaptosomes and cell cultures have been used to study tryptophan uptake into brain cells. As with transport at the blood-brain barrier, transport at this membrane is composed of a saturable and non-saturable component. In addition, the system is shared by large neutral amino acids which compete for uptake (Kiely and Sourkes, 1972; Grahame-Smith and Parfitt, 1970). Uptake at the brain cell membrane has been well reviewed by Young and Sourkes (1977). Movement of amino acids in and out of the cerebrospinal fluid (CSF) has been studied, but to a relatively minor extent. Amino acids can gain access to the CSF directly by being secreted from the extracellular fluid across the ependymal or pial membranes. CSF levels of amino acids are substantially lower than plasma levels in man (Hagenfeldt et al., 1984), dog (Bito et al., 1966) and rat (Franklin et al., 1975). There is a decreasing concentration gradient from the plasma to extracellular fluid to CSF (Bito et al., 1966). The lower levels of amino acids in the CSF are thought to be due to the active transport of amino acids from CSF to blood. The site of transport in mammals is probably the choroid plexus, though the arachnoid membrane may also be involved (Lorenzo, 1974) as has been found in the frog (Wright, 1978). Amino acids administered into the subarachnoid

space were found to penetrate cerebral tissue (Lajtha and Toth, 1962). Bidirectional movement of glycine between blood and CSF was demonstrated in the rat (Franklin et al., 1975) but ventricular-cisternal perfusion did not result in a substantial loss of amino acids from the CSF in the cat (Levin et al., 1966). An in vitro experiment using a model amino acid for the small neutrals found that there was an active transport system in the choroid plexus in the cat (Lorenzo and Cutler, 1969). Despite differences in experimental design and final conclusions, it is generally accepted that there is an active transport system for amino acids out of the CSF, the specifics of which are unknown. Of importance to three of the studies in this thesis is the fact that CSF tryptophan changes in parallel with CNS tryptophan and is thus useful index of the brain levels of this amino acid (Young et al. 1976).

1.2.4 Physiological factors which influence brain tryptophan

Tryptophan is the only amino acid that is transported in the blood bound to albumin (McMenamy et al.,1957). Of the total tryptophan found in the plasma, 80-90% is bound while the rest is free. There is one tryptophan binding site per albumin molecule (McMenamy and Oncley, 1958). This binding site also binds nonesterified fatty acids (NEFA) (McMenamy, 1964) and is the primary binding site for medium-chain fatty acids (Cunningham et al., 1974). In humans, a direct correlation exists between plasma NEFA and non-albumin bound (NAB) tryptophan (Curzon et al., 1974). Therefore the amount of NAB tryptophan depends not only on the concentration of albumin and tryptophan in the plasma but also on the NEFA concentration. Drugs which increase unesterified fatty acids will increase NAB tryptophan as well. This has been demonstrated with heparin (Knott and Curzon, 1972), noradrenaline, L-DOPA, and aminophylline (Curzon and Knott, 1974). Plasma NEFA are derived from

lipolysis of body fat, a process stimulated by increases in cyclic AMP levels (Brodie et al., 1969). Thus, experimentally induced stress in animals such as food deprivation and immobilization increases NAB tryptophan but has no effect on total tryptophan, i.e. albumin bound plus NAB tryptophan (Knott and Curzon, 1972). The removal of group-housed rats from their cages, a mild psychological stress, will increase NAB tryptophan in fasted rats, an effect which can be inhibited with the antilipolytic drugs, propanolol and nicotinic acid (Curzon and Knott, 1975).

Though immobilization and food deprivation were not found to alter plasma total tryptophan, both treatments increased brain levels (Curzon et al., 1972). The finding that the same treatments increased NAB tryptophan and brain tryptophan (Knott and Curzon, 1972) led to an extended controversy on whether NAB tryptophan or the ratio of tryptophan to the sum of the competing amino acids was the best predictor of changes in brain levels. It was argued that tryptophan bound to albumin was not available for uptake at the blood-brain barrier (Knott and Curzon, 1972; Fernando et al., 1976). Using the Oldendorf technique, albumin was found to decrease the brain uptake index of tryptophan, suggesting that the binding of tryptophan to albumin can inhibit tryptophan uptake. However, inhibition of uptake by albumin was not as large as would be expected if only the NAB tryptophan was available to the brain. This suggests that some of the albumin-bound tryptophan is available for uptake (Etienne et al., 1976; Yuwiler, 1977).

In contrast, Madras et al. (1974) showed that while increasing the concentration of fat in the diet increased NAB tryptophan, no significant

changes were observed in brain tryptophan. They suggested that if the affinity of the brain transport site for tryptophan was greater than the affinity for albumin, then tryptophan could be stripped off the protein and available for transport. This concept was supported by Pardridge (1979) who found that the high apparent binding capacity of the BBB enables the capillary transport system to compete with albumin for tryptophan binding. He proposed that tryptophan availability dependent on five parameters; albumin concentration, rate of [illegible] tryptophan transport system, capillary transit time, affinity [illegible] for tryptophan (which is dependent on plasma NEFA concentration) and the affinity at the BBB which depends on the concentration of the other large neutral amino acids.

Certainly, it is true that, in some experimental paradigms, brain levels are correlated with free plasma tryptophan, while in others the ratio is a more appropriate indicator (Curzon and Sarna, 1984). For example, in rats made diabetic by streptozotocin, plasma levels of the branched chain amino acids are very high due to lack of insulin (see next section). Subsequently, there is increased competition for uptake ,less tryptophan can enter the brain, thereby lowering brain tryptophan (Crandall and Fernstrom, 1983). Thus, tryptophan uptake is determined by the ratio of tryptophan to the sum of the competitors. A contrasting situation is exhibited by the effect of exercise on brain tryptophan in rats. Two hours of running had no effect on total plasma tryptophan or on plasma concentrations of the competing amino acids, except for a slight increase in leucine. A significant elevation was observed in both NAB tryptophan and brain tryptophan indicating that under these experimental conditions tryptophan availabilty is determined by free

tryptophan (Chaouloff et al., 1986a). Other manipulations require that both factors be considered, such as in the genetically obese Zucker (fa/fa) rats, which exhibit lowered free tryptophan, increased concentrations of the competitors and decreased brain tryptophan as compared to heterozygous controls (Fa/fa) (Finkelstein et al., 1982). The authors concluded that the degree of decline in brain tryptophan corresponded better with the ratio than with NAB tryptophan.

Interestingly, the original hypothesis suggesting that the rise in brain tryptophan after immobilization is mediated by free tryptophan has recently been qualified. Kennett et al. (1986) found elevated brain levels of the other neutral amino acids (except histidine and methionine) after two hours of restraint stress. Brain influx calculated from plasma levels, using the method of Pardridge and Oldendorf (1975) were not found to be altered. They suggested that the increase in brain tryptophan is not due to free plasma tryptophan but the result of a common mechanism which is responsible for the increase in brain amino acid levels. It was postulated that the kinetics of transport across the blood-brain barrier were altered.

1.2.5 Effect of acute dietary intake on the plasma tryptophan ratio and/or brain tryptophan in animals.

As demonstrated in the preceding sections, the relationship between plasma and brain levels of tryptophan is complex. A discussion concerned with the effects of diet on brain tryptophan must include the same factors which are important in uptake, such as availability of tryptophan and concentration of the competing amino acids as well as dietary components and physiological consequences of food consumption. This

section will examine the effects of acute dietary intake on plasma and brain tryptophan, reflecting the focus of the experimental work presented in the following chapters.

Direct correlations between diet, plasma and brain levels of tryptophan are seen only in extreme manipulations of dietary intake. Hence, supplementation of a whole egg protein diet with 0.22 or 0.32% tryptophan will increase serum and brain levels of tryptophan, when measured two hours later (Yokogoshi and Wurtman, 1987). Alternatively, the acute administration of a diet deficient in tryptophan but containing all the other essential amino acids results in a 90% decrease in RNA tryptophan, a 75% decrease in total tryptophan and a 90% decline in brain levels (Biggio et al., 1974). In both experiments, the animals had been trained to consume their food within a two hour period. The extreme depletion of tryptophan observed after the ingestion of the tryptophan deficient diet is due to the removal of free levels of the missing amino acid as it is incorporated into protein.

Ingestion of more natural diets, or diets containing pure dietary constituents, do not lead to direct correlations between dietary content of tryptophan, plasma and brain levels. A diet containing only carbohydrate and therefore no tryptophan, will increase both plasma and brain tryptophan in rats, from one to three hours after ingestion (Fernstrom and Wurtman, 1971b). This is primarily due to the overall anabolic effect of insulin on energy metabolism. After carbohydrate ingestion, the insulin released is instrumental in the uptake of amino acids as well as glucose into the cells (Lotspeich,1949). The branched chain amino acids are taken up to a greater extent than the aromatic amino acids. In addition, NEFA are taken up for lipogenesis (Tepperman,

1980). The uptake of the branched chain amino acids decreases the concentration of the competing amino acids in the plasma, thereby increasing the ratio of tryptophan to the sum of the competitors (the plasma trytophan ratio) (Fernstrom and Wurtman, 1971b; Fernstrom and Faller, 1978; Peters and Harper, 1987; Leathwood, 1987). The decrease in NEFA, allows more tryptophan to bind to albumin, resulting in an increase in total tryptophan and a decline in NAB tryptophan (Madras et al., 1973). Thus, the increase in total tryptophan combined with the decline in the competing amino acids, results in the paradoxical effect of a carbohydrate diet increasing brain tryptophan levels. A simultaneous increase in brain influx, as calculated from the equation developed by Pardridge and Oldendorf (1975), has also been reported (Fernstrom and Faller, 1978).

It is important to note that in all experiments described above, the animals had been fasted for extended amounts of time. Fasting (section 1.2.4) increases brain tryptophan due to an increase in NAB tryptophan (Knott and Curzon, 1972). It has been suggested that the greater the elevation of brain tryptophan due to fasting, the smaller the difference that will be observed after carbohydrate, due to the fall in NAB tryptophan (Sarna et al., 1985). This could be a possible explanation for the failure of a carbohydrate diet to raise brain tryptophan in one study (Glaeser et al., 1983). An additional factor common to these dietary experiments is the inclusion of fat in the carbohydrate diet. Madras et al. (1973) found that though the addition of fat to carbohydrate attenuated the decrease in NAB tryptophan and therefore the increases in total tryptophan, it did not significantly effect brain levels of tryptophan. Similarly, other work has shown that though fat

can effect other aspects of metabolism, brain tryptophan is unaltered (Brindley et al., 1984; Teff and Young, unpublished results). Glucose alone or subconvulsive doses of insulin can also increase brain tryptophan (Madras et al., 1973).

Protein consumption increases plasma tryptophan (Fernstrom and Faller, 1978; Glaeser et al., 1983; Peters and Harper, 1987) but to a much smaller degree than the increase of other amino acids. This is because tryptophan is the least abundant amino acid in protein. The large increase in competing amino acids observed after protein ingestion not only prevents an increase in the tryptophan ratio but in some cases results in a decrease. Thus, instead of an increase in brain tryptophan, as one would expect after a protein meal, levels either remain unchanged (Fernstrom and Faller, 1978) or decrease with respect to fasted controls (Peters and Harper, 1987; Glaeser et al.,1983). The administration of five large neutral competing amino acids to a carbohydrate-fat diet significantly decreased the plasma tryptophan ratio and brain tryptophan. When a mixture of all the amino acids excluding tryptophan and the neutral amino acids were added to the carbohydrate-fat diet, the rise in brain tryptophan was still observed (Fernstrom et al., 1975). A direct relationship between the plasma tryptophan ratio and brain tryptophan after the consumption of single meals either varying in protein or amino acid content, have been reported by Fernstrom and Wurtman (1972), Fernstrom and Faller (1978) and by Leathwood (1987). Despite these findings and though changes in the tryptophan ratio are generally associated with changes in brain levels, in some studies no changes in brain tryptophan were seen. Fernstrom and Faller (1978) found an increase in the ratio after an 18% protein diet with a corresponding

increase in influx (as calculated using the Pardridge and Oldendorf equation, 1975) but brain tryptophan levels remained the same. Peters and Harper (1987) observed a decrease in brain tryptophan after administration of 5% protein despite a slightly elevated tryptophan ratio. In free-feeding animals that had not been fasted, protein had no effect on brain tryptophan (Leathwood, 1987).

Various questions arise as to whether the quantity or quality of protein can differentially effect brain tryptophan. In the experiments described above, rats were fasted and then given a diet that contained protein, fat and carbohydrate. In three of the studies (Peters and Harper, 1987; Glaeser et al., 1983; Fernstrom and Faller, 1978) fat was kept constant while protein and carbohydrate were varied to keep the diets isocaloric. Fernstrom and Faller (1978) found 18% protein resulted in elevated ratio levels with respect to fasted animals while 40% protein had no effect. Brain levels were unaltered by either treatment. Glaeser et al. (1983) found a decrease in brain tryptophan two hours after the 18% and 40% diet (as in the above experiments, fat was held constant and the other maconutrients were varied), though a significant difference between the two amounts of protein was not noted. The plasma tryptophan ratio was not calculated in this experiment. A significant decrease in brain tryptophan was reported after the administration of 5% casein with respect to fasted animals (Peters and Harper, 1987). In this experiment, different levels of protein were administered ranging from 5-55%. Unfortunately due to the graphical presentation of the material and the lack of statistical variation reported, it is difficult to interprete differences between protein levels on brain tryptophan. Yokogoshi and Wurtman (1986) systematically varied the protein and carbohydrate content

of the diet. They found that as little as 5% casein added to a 70% dextrose and 10% fat diet inhibited the rise in the tryptophan ratio. An inverse relationship between protein content and the tryptophan ratio was not observed. Interestingly, various types of protein exhibited different degrees of inhibition of the rise in the ratio. This was thought to be a reflection of their amino acid content. Therefore, there is no evidence that increasing the protein content of a meal will proportionately decrease the tryptophan ratio or brain tryptophan. In fact, it seems that significant changes in the ratio will occur only after the ingestion of a pure carbohydrate meal or upon administration of a meal containing protein, after the animal has been fasted. In some cases, brain tryptophan is altered and in others, it is not. What accounts for the discrepancies observed between the different studies? One factor which must be considered is the extent to which the ratio must be altered in order to result in an alteration of brain tryptophan. Approximately, a four-fold change in competitor concentration leads to a 2 fold change in both tryptophan influx or brain tryptophan (Curzon and Sarna, 1984). Ashley et al. (1985b) estimate that a 50-100% rise or a 30-50% fall in the plasma tryptophan ratio is necessary to alter rat brain tryptophan levels. Therefore the magnitude of the change in the tryptophan ratio is an important determinant of brain tryptophan.

In conclusion, both protein and carbohydrate can influence brain tryptophan in animals but there are a number of variables which can either attenuate or enhance the dietary effects.

1.2.6 Effect of dietary intake on the plasma tryptophan ratio in humans.

Many of the factors and variables that are relevant in animals

with respect to dietary effects on the plasma tryptophan ratio, are pertinent to humans as well. The primary difference is the emphasis placed on the ratio as an index of tryptophan availability to the brain. The lack of access to human brain metabolism results in the use of indirect measurements to determine effects of dietary intake. Traditionally, these have been either plasma amino acid levels or behavioral measures as a means of assessing function. This section will discuss the biochemical aspect, while chapter 1.4.3 will cover effects on a behavior thought to be mediated by 5HT, macronutrient selection.

The same physiological factors which regulate the effects of protein and carbohydrate on the plasma tryptophan ratio in animals, are also influential in humans. Hence, insulin, as a secondary response to glucose intake, lowers plasma amino acid levels (Zinneman et al., 1966; Adibi et al., 1975). Branched chain amino acids were found to decrease to a greater extent than the aromatics. A dose-dependent, inverse relationship was observed between glucose intake and the large neutral amino acids, two hours after administration. Twenty-five and 50g of glucose significantly increased the plasma tryptophan ratio, by approximately 17 and 21%, respectively (Martin-Du Pan et al., 1982). Though glucose administration was found to decrease NAB tryptophan, increases in total tryptophan were not observed (Lipsett et al., 1973). This is one response in which humans differ from animals, as rats exhibit an increase in total tryptophan after glucose (Madras et al., 1973). The magnitude of change in the tryptophan ratio seen in humans after carbohydrate is significantly smaller than the 3-4 fold increases reported in rats. This difference can be accounted for in part by the unresponsiveness of total tryptophan to carbohydrate in humans.

Ingestion of pure protein, such as 25g of albumin increases plasma tryptophan and decreases the tryptophan ratio by about 50%. Smaller amounts of protein (6 and 12.5g) also decreased the ratio but the effect of 6g was of shorter duration (Moller, 1985). Administration of standard diets or diets cotaining both macronutrients have led to contradictory results. A carbohydrate diet containing 6% protein, given in the evening, did not significantly increase the tryptophan ratio (Ashley et al., 1982). Nor did a diet containing 20% protein lower the ratio. In contrast, significant effects were reported with both meals when they were given in the morning after an overnight fast. The carbohydrate meal increased the tryptophan ratio by approximately 15%, while the protein meal lowered it 27% within 60 minutes of treatment (Ashley et al., 1982). Therefore, as has been discussed in the animal experiments, the nutritional state of the subject is an important variable in determining the outcome. Perez-Cruet et al. (1974) administered two isocaloric standard diets and measured the plasma neutral amino acids . The first blood sample was taken after a 24h fast in the morning and the second, four hours after ingestion of the test meals. They did not find a significant decline in the ratio of total tryptophan to the sum of the competing neutral amino acids but instead a 50% decrease in the ratio of free tryptophan to the sum of the competitors, with respect to the fasted controls.

Therefore in conclusion, one important question is raised from the human literature; Can acute intake of normal dietary constituents alter tryptophan availability to the extent that brain tryptophan and 5-HT are altered as well?

1.3.1 5-hydroxytryptamine, the neurotransmitter.

1.3.2 Synthesis and metabolism of 5-hydroxytryptamine in the central nervous system

5-hydroxytryptamine (5HT) or serotonin is thought to be a neurotransmitter in the mammalian central nervous system. One percent of whole body 5HT is found in the brain, where the cell bodies of 5HT neurons lie within the mesencephalic and medullary raphe nuclei. Axons project down into the spinal cord and up into the mid- and forebrain (Dahlstrom and Fuxe, 1964). High concentrations of 5HT are found in the hypothalamus, particularly the superchiasmatic nucleus, of the rat (Lookingland et al., 1986). The pons, medulla and the midbrain also contain fairly high concentrations of 5HT in the dog and the rat (Moir and Eccleston, 1968; Knott and Curzon, 1974). The concentration of the amine in a specific brain area does not necessarily correlate with the responsivity of the area to various treatments which effect metabolism (Moir and Eccleston, 1968; Knott and Curzon, 1974).

The synthesis of 5HT in the CNS occurs within serotonergic neurons. Two enzymatic reactions are required. The first involves the hydroxylation of tryptophan to 5-hydroxytryptophan (5HTP) which is then decarboxylated to form 5HT. Inactivation of 5HT occurs when the neurotransmitter is converted to its primary metabolite, 5-hydroxyindoleacetic acid (5HIAA), by the enzymes monoamine oxidase (MAO) and aldehyde dehydrogenase.

The first and rate limiting enzyme (Ashcroft et al., 1965; Moir and Eccleston, 1968) is tryptophan hydroxylase, which is found only in 5HT neurons. Tryptophan hydroxylase is a mixed function monooxygenase enzyme

requiring oxygen and biopterin as cofactors. The natural cofactor is thought to be L-erythro-tetrahydrobiopterin (BH4). Different pterin cofactors can alter the affinity of the enzyme for tryptophan. Though not confirmed, some evidence indicates that activation of tryptophan hydroxylase can occur by a Ca-calmodulin dependent protein kinase, resulting in a concommitant decline in the Km of the enzyme for biopterin (Nagatsu, 1983). Relatively little is known about the mechanism of regulation of tryptophan hydroxylase in comparison with the other monooxygenase enzymes such as tyrosine or phenylalanine hydroxylase. This is because of the difficulty in purifiying the enzyme and its relatively low activity. Unlike tyrosine hydroxylase which exhibits substrate inhibition at low levels of substrate, tryptophan hydroxylase in vivo does not show this property (Grahame-Smith, 1971). Electrical depolarization of brain tissue slices resulted in an increased affinity of the enzyme for tryptophan independent of precursor concentration (Elks et al., 1979).

Substantial experimental evidence suggests that tryptophan hydroxylase is unsaturated with tryptophan at normal physiological concentrations. This would imply that 5HT synthesis is dependent in part on tryptophan availability. The concentration of the precursor is not the sole factor regulating the synthesis of 5HT, but it is the one of primary interest with respect to the effects of dietary intake on brain 5HT. Other factors which control 5HT synthesis, and are out of the scope of this thesis, are discussed by Mandell et al. (1974) and Kuhn et al. (1986).

Evidence for the unsaturation of tryptophan hydroxylase was first shown by Hess and Doepfner (1961) who administered tryptophan to rats

pretreated with a monoamine oxidase inhibitor (MAOI) and reported an increase in brain 5HT. Tryptophan alone was also shown to increase brain levels of 5HT but 5HTP was barely detectable (Ashcroft et al., 1965). These findings suggest that tryptophan hydroxylase is the rate limiting enzyme in 5HT synthesis and also that the enzyme is unsaturated with tryptophan. Studies in tryptophan loaded rats on the accumulation of 5HT after inhibition of monoamine oxidase (Grahame-Smith, 1971) and accumulation of 5HTP after inhibition of aromatic amino acid decarboxylase (Carlsson and Lindqvist, 1978) suggest that the enzyme is normally 50% saturated with substrate. Physiological amounts of tryptophan as low as 5% of the daily dietary itake of the rat can significantly increase brain 5HT, while larger amounts can double brain 5HT levels (Fernstrom and Wurtman, 1971a).

In vitro measurements of the affinity of tryptophan hydroxylase for tryptophan found the Km to be 50uM (Friedman et al., 1972), which would also suggest that the enzyme is half saturated, as the brain content of tryptophan is around 30uM. Others have reported somewhat different Km's in rat brain; 20 uM (Ichiyama, 1970), 25uM (Carlsson and Lindqvist, 1978), and 14uM (Kuhn et al., 1986). As the Km of the enzyme for substrate can be altered by the type of pterin cofactor used, this interjects a confounding variable in exact determination of the affinity of the enzyme. Similarly, lack of homogeneity in the concentration of tryptophan in the brain adds to the difficulty in determining the degree of saturation. Not only do tryptophan levels vary depending on the specific brain area, but tryptophan content in whole brain does not necessarily correspond to levels within the synaptosome. Therefore, it is difficult to conclude to what extent tryptophan hydroxylase is

saturated under normal conditions, from the evidence obtained in vitro.

Experimental treatments which lead to changes in both tryptophan and 5HT lend support to the argument that 5HT levels are dependent on tryptophan availability. Hydrocortisone decreases brain tryptophan by 40% and brain 5HT by 21% (Green et al., 1975). Alpha-methyltryptophan decreases both compounds as well (Sourkes, 1971). Both immobilization and food deprivation increase brain tryptophan, which was found to correlate with brain 5HT levels (Curzon et al., 1972). Occasionally, treatments increase brain tryptophan and brain 5HIAA levels without altering brain 5HT (Perez-Cruet, 1972; Curzon et al., 1972; Knott and Curzon, 1972), suggesting an increased turnover of neurotransmitter resulting in an increased level of the metabolite.

Measurement of the metabolite, 5-hydroxyindoleacetic acid has often been used as an index of both turnover i.e. synthesis and metabolism of 5HT (Neff, 1972), and release. It has been assumed that 5HIAA levels in the brain are a direct result of released 5HT that has been taken back up into the neuron and metabolized by the mitochondrial-bound enzyme, monoamine oxidase. Behavioral and neurochemical data suggest that 5HIAA levels may in some circumstances be more a reflection of intraneuronal metabolism of 5HT that has not been released, than of 5HT release. Administration of tryptophan will increase brain 5HT, but there is a much larger elevation of 5HIAA levels (Eccleston et al., 1965). When monoamine oxidase is inhibited, the increase in 5HT will be much greater. The first treatment has no gross behavioral effects in rats, but the second is associated with a hyperactivity response. It has been suggested that when tryptophan is given alone, the extra 5HT that is synthesized will be metabolized intraneuronally. Alternatively, if MAO

is inhibited, then the surplus neurotransmitter will spill over, independent of neuronal firing, and is available for interaction at post-synaptic receptor sites, causing a behavioral change (Green and Grahame-Smith, 1976). Similarly, Wolf et al. (1985), using a combination of drug treatments and behavioral observations, concluded that 5HT can be deaminated within the neuron before release.

1.3.3 Functional 5-hydroxytryptamine

Ultimately, the goal of any experiment is to determine if the changes observed as a result of the treatment are of physiological or pharmacological, i.e. functional, significance. Measuring brain levels does not discriminate between intra- and extracellular compartments nor between stored and active pools of the transmitter in question. Increased synthesis, which would elevate brain levels, does not necessarily mean that an increased amount of neurotransmitter is released. Alternatively, decreased brain levels may not correspond to a decreased release. The amount of transmitter that is released and is available for interaction with post-synaptic receptors must be considered as the functionally active component.

Tryptophan availability is only one of the factors that is influential in the amount of 5HT released. Neuronal firing (Elks et al., 1979), MAO activity, reuptake of 5HT and regulation of release by autoreceptors (Brazell et al., 1985) will determine the final concentration in the synaptic cleft. Advancements in evaluating functional activity have been hampered by methodological problems. One approach is to measure neurotransmitters in extracellular fluid (ECF). However, only small volumes of ECF can be obtained. This and the low

concentrations of neurotransmitters causes analytical problems. Several techniques have been employed in the measurement of compounds related to 5HT metabolism. These include: push-pull cannula, usually coupled with either a radioenzymatic assay, high performance liquid chromatography (HPLC) (Ruwe et al., 1985) or isotopic precursor labelling (Herv et al.,1979), in vivo dialysis coupled with HPLC (Hutson et al., 1985), in vivo amperometry (Marsden et al.,1979) and measurement of compounds in the CSF (Hutson et al.,1985; Chaouloff et al.,1986b). The last method will be addressed in the following section. Push-pull cannula and in-vivo dialysis involve the insertion of a probe, perfusion with artificial CSF and collection of either the perfusate or dialysate. These techniques have the advantage of specificity and sensitivity. Unfortunately, brain tissue and hence capillary damage can result, thereby releasing 5HT from platelets which can contaminate sampling (Ruwe et al., 1985) . In the amperometric techniques, signal verification is accomplished by administration of drugs which act on 5HT such as; fluoxetine, p-chloroamphetamine (Marsden et al., 1979, Joseph and Kennett, 1981) and p-chlorophenylalanine (De Simoni et al., 1987), but neither voltammetry nor in vivo electrochemical detection have the specificity to distinquish between 5HT and 5HIAA (Echizen and Freed, 1986; Joseph and Kennett, 1981; De Simoni et al., 1987). Other non-amine compounds may interfere with the signal as well (Mueller et al. 1985; Adams, 1986). Despite these drawbacks, pertinent information concerning extracellular 5HT can be derived.

The effect of tryptophan loading on extracellular 5-hydroxyindoles was examined using in vivo voltammetry. In the striatum of unanaesthetized rats (Marsden et al., 1979; De Simoni, 1987), tryptophan

administration did not increase the electrochemical signal. When the electrode was implanted in the dorsal hippocampus, an area responsive to tryptophan administration (Knott and Curzon, 1974), a dose-dependent increase in the current was observed (Joseph and Kennett, 1981). If the animals were treated with pargyline, a MAO inhibitor then a small increase of current in the striatum was observed (Marsden et al., 1979). Immobilization stress which increases brain tryptophan and 5HT (section 1.2.4), also increases the electrochemical signal in the hippocampus, an effect which can be attenuated by pretreatment with valine, one of the competing amino acids (Joseph and Kennett, 1983). As previously discussed, this technique does not differentiate between 5HIAA and 5HT, therefore it is possible that the signal is derived from 5HT metabolized intraneuronally and not that which is functionally active. One of the few experiments that measured 5HT directly showed an increase in release of radioactive 5HT after an acute tryptophan injection containing labelled tryptophan and also following constant intravenous perfusion (Ternaux et al., 1976). Similarly, in superfusate collected from the ependymal surface of the caudate nucleus of the encephale isole cat, tryptophan adminstration produces a maximal increase of 5HT one hour following injection (Ternaux, 1977). Using an in vivo dialysis probe in the striatum, an increase in tryptophan and 5HIAA was found after a tryptophan load (Hutson et al., 1985).

Another approach to the question of functionally active 5HT has been to monitor the firing rate of 5HT neurons electrophysiologically. The activity of serotonergic neurons is known to correlate with the level of behavioral arousal (Trulson and Jacobs, 1979). It has been suggested that tonic changes in motor activity and/or the tonic level of activity

in the peripheral and central nervous system can influence the discharge rate of brain 5HT (Jacobs, 1986). One study examined the acute effect of three different diets on the activity of single 5HT-containing neurons in the dorsal raphe (Trulson, 1985). Two experiments were performed on separate groups of animals which were food deprived for twenty-four hours and then administered one of three diets; standard chow diet, a diet containing a normal amount of tryptophan but lacking in the competing neutral amino acids and a tryptophan-free diet containing the competitors. The first experiment was designed to determine if the activity of the neurons could be altered by dietary treatment. Unit acitivity was recorded for four hours after administration of the diet. No significant differences in activity were found. A transient increase was observed almost immediately after feeding though this change in the activity of the 5HT-containing neurons was most likely due to the arousal state after food presentation. Tryptophan can inhibit firing of serotonergic neurons (Gallagher and Aghajanian, 1976), therefore it is possible that increases in brain tryptophan will inhibit the release of 5HT. The second experiment (Trulson, 1985) considered the possibility that the rate of firing activity may not change but that the functional concentration of 5HT may be altered. Therefore, labelled tryptophan was perfused into the lateral ventricle two hours after presentation of the different diets. One hour later, perfusion was peformed. Differences due to the three treatments in the amount of released, labelled 5HT were not demonstrated, though there was an increase in the amount of 5HIAA released in the animals which consumed the diet lacking the competitive amino acids. A decrease in the metabolite was reported in the tryptophan-free diet. It is possible that changes in functional 5HT were not seen in the labelled 5HT, which would come primarily from the newly

synthesized pool of 5HT. Alternatively, the changes may have occurred earlier than during the three to four hour time period after dietary intake at which time the experiment took place.

One can conclude that under certain circumstances, changes in functional 5HT can be demonstrated after tryptophan loading. However, as only one study has examined the effect of diet on functionally active 5HT, it is to early to draw a conclusion concerning the question of whether dietary components can alter the amount of 5HT released.

1.3.4 Tryptophan, 5HT and 5HIAA in cerebrospinal fluid

The measurement of tryptophan, 5HT and 5HIAA in cerebrospinal fluid (CSF) is one of the few ways of assessing CNS metabolism in humans . Abnormal levels of tryptophan or 5HIAA in CSF are thought to be associated with pathological states. Depressive (Coppen et al.,1972), suicidal (Cronholm and Asberg, 1977) and aggressive (Brown et al., 1982) patients usually manifest low CSF 5HIAA. Several questions have been raised with respect to the use of these componds as an index of CNS metabolism and function, for example; Are compounds in the CSF derived from peripheral or central metabolism? Do changes in the CSF reflect changes in the brain?, Is 5HIAA in the CSF an index of released 5HT? Animal studies have attempted to answer some of these questions but until recently, direct quantitation of 5HT was not possible and the majority of experiments were only able to determine tryptophan and 5HIAA. This section will examine the use of CSF tryptophan, 5HT and 5HIAA as indices of brain metabolism and function.

As there is no significant penetration of peripherally administered 5HIAA into the CSF (Ashcroft et al., 1968), it is assumed the metabolite must be derived from the central nervous system. The exact origin of

5HIAA within the CNS is still not known as evidence is contradictory concerning the relative contributions from the brain and spinal cord to lumbar CSF 5HIAA (Garelis et al., 1974). The existence of a ventricular-lumbar gradient supports the hypothesis that a proportion of lumbar CSF 5HIAA is from the spinal cord (Moir et al., 1970; Gillman, 1981; Stanley et al., 1985). The lag observed in the increase of CSF 5HIAA after tryptophan administration suggested that the spinal contribution was small, as this time might be a reflection of the diffusion of the metabolite down from higher levels (Eccleston et al., 1970). It has been estimated that at most 40% of lumbar CSF 5HIAA is of spinal origin (Garelis et al., 1974).

In animal studies, correlations can be made between tissue and CSF levels, which are usually altered in the same direction after various treatments. Thus, administration of chlorpromazine to dogs decreased 5HIAA in the caudate and in ventricular CSF, maintaining the same ratio as observed in control animals (Moir et al, 1970). Alpha-methyltryptophan which lowers brain levels of tryptophan in rats, significantly reduced CSF tryptophan and 5HIAA, suggesting that CSF 5HIAA can vary with the availability of the precursor. This is substantiated by the effect of the administration of an amino acid mixture deficient in tryptophan which lowers both CSF tryptophan and 5HIAA (Young et al., 1980).

Increases in precursor availability can also influence CSF levels of tryptophan and 5HIAA. Modigh (1975) added tryptophan to a standard rat chow containing no free tryptophan and found significantly correlated

increases in brain and CSF tryptophan and 5HIAA in the animals receiving the supplemented diet. When tryptophan was injected intraperitoneally, an increase was observed one hour after treatment in rat CSF tryptophan and 5HIAA (Young et al., 1980). In contrast to the experiments described above which were performed on animals which were anesthetized for CSF sampling, Hutson et al. (1985) implanted a chronic indwelling catheter into the cisterna magna. This enabled them to withdraw CSF from conscious freely moving animals. A 15 uL CSF sample was collected every 20 minutes. Tryptophan loading (i.p) increased CSF tryptophan by approximately 500%, while 5HIAA was elevated 102%, a finding consistent with the hypothesis that normally tryptophan hydroxylase is half saturated. Time to peak concentration was 46+10 minutes and 72+17 minutes for each compound respectively. A large degree of interanimal variability was evident, despite the number of factors which were held constant such as strain of animal, diet, housing and time of day. The large individuality in CSF tryptophan tends to overide the effect of more subtle treatments. Glucose administration which increased brain tryptophan was found to increase CSF tryptophan as well, though this effect was not significant due to the variability in the CSF results (Young et al., 1976). Two physiological manipulations have been shown to alter CSF levels of tryptophan in conjunction with changes in brain tryptophan. In one experiment, elevated CSF tryptophan was reported in rats starved for 24 hours (Young et al., 1976). In the other, elevated motor activity increased CSF tryptophan and 5HIAA in an experiment in which CSF was withdrawn from the third ventricle of conscious rats (Chaouloff et al., 1986b). The effect of dietary intake on CSF tryptophan, 5HT or 5HIAA has not been studied in any animal experiments.

In humans, only one post-mortem study examined the relationship between tissue and CSF concentrations. They demonstrated a significant correlation between levels of 5HIAA in the cerebral cortex and CSF (Stanley et al., 1985). A similar relationship was observed after infusion of tryptophan in psychiatric patients undergoing a stereotactic subcaudate tractotomy. Increased tryptophan concentrations in the frontal cortex and both lumbar and ventricular CSF were found. 5HIAA levels were increased in the two fluid compartments as well (Gillman et al., 1981). The effect of tryptophan on CSF tryptophan and 5HIAA had been demonstrated in an earlier study whereby oral ingestion of tryptophan (50 mg/Kg) increased CSF tryptophan two hours after administration, while the rise in CSF 5HIAA began at four hours (Eccleston et al., 1970). Three grams of tryptophan, which corresponds to approximately twice the daily dietary intake, was found to double 5HIAA levels in CSF but 6 grams caused no further increase (Young and Gauthier, 1981). This suggests that, in humans, tryptophan hydroxylase must possess the same degree of saturation as is observed in animals.

One study has examined the effect of diet on CSF tryptophan and 5HIAA (Perez Cruet et al., 1974). Two balanced isocaloric diets were given, differing only marginally in protein content. The patients were fasted for 24 hours, with one lumbar puncture done in the morning and the second, four hours after lunch was eaten. A 20% decrease in both CSF tryptophan and 5HIAA was reported after ingestion of one of the diets. The other diet caused a 21% decrease in CSF tryptophan and a 35% decrease in CSF 5HIAA. This decline was associated with a significant decrease in the ratio of free plasma tryptophan to the sum of the competing amino acids.

In a number of the studies cited above, changes were observed in the precursor and the metabolite of 5HT. If both are indices of 5HT metabolism then there should be some relationship between the two compounds. In rats, a significant correlation was found between CSF tryptophan and 5HIAA after a tryptophan supplemented diet (Modigh, 1975). Similar results are seen in humans after tryptophan loading (Ashcroft et al., 1973). Under normal conditions, some groups have reported significant correlations (Curzon et al., 1974) while others have not (Young et al., 1974a). As tryptophan availability is only one of the factors controlling 5HT synthesis, the correlation between tryptophan and 5HIAA would not be exected to be large and would be seen only with large sample sizes.

Recently some laboratories have reported the measurement of 5HT in the CSF of normal and psychiatric patients. In normal controls, values have been reported as 80 pg/ml (Tyce et al.,1985), 12 ng/ml (Volicer et al., 1985) and 5 pg/ml (G.M. Anderson, personal communication). For psychiatric disorders, an average of 1 ng/ml was reported for unipolar depressives (Linnoila et al., 1986) and 93-902 pg/ml for a group of Parkinsonian and Gilles de la Tourette patients (Artigas et al., 1985). Though obviously the measurement of 5HT is more informative of functional 5HT than 5HIAA, the wide discrepancy in control values makes interpretation of clinical studies impossible.

Measurement of tryptophan, 5HT or 5HIAA in CSF, like most techniques, has inherent advantages and disadvantages. In humans, the relative non-invasiveness of the technique allows some degree of access to the central nervous system which is normally not available. Several factors are known to influence the concentration of 5HIAA in human lumbar CSF. Motor activity, site of puncture, volume of CSF withdrawn, age, sex, body height can all influence levels of the metbolite and must be standardized

within each experiment (Bertilsson, 1987). In animals, withdrawal of CSF from the cisterna magna, the site of removal of most experiments described above, is relatively simple and unlikey to result in contamination by platelets. On the other hand, one obvious disadvantage is the lack of neuroanatomical specificity. Determination of a compound in the CSF reflects overall changes in the brain, not a specific brain area. If a treatment is likely to be effective at a specific location, it is unlikely that a small change could be detected due to the large variability in compounds in the CSF. However, the focus of the experimental work presented is to examine the effect of diet on brain metabolism and at the present time there is no evidence to suggest that dietary effects on the brain which are mediated by changes in neurotransmitter precursor availability are specific to one area.

1.3.5 5HT in the peripheral nervous system:localization and physiology

The majority of 5HT in the whole animal is located in peripheral tissues, which lie outside the central nervous system. Only 1% is actually contained within the brain. 90% is found in the intestine and approximately 8% in blood platelets. Small quantities also exist in the lung, heart, liver,stomach, spleen, pancreas and pineal (Twarog and Page, 1953; Bender et al., 1975). Though it is beyond the scope of this thesis to examine the effects of 5HT in each of these tissues, its physiological role in three of the tissues; intestine, pancreas and pineal will be reviewed briefly.

5HT is found throughout the gastrointestinal tract, primarily

in the enterochromaffin cells (EC) of the intestinal mucosa, where it is co-localized with a number of polypeptides. The enterochromaffin cells are innervated by both the vagal and splanchnic nerves which can modulate release of 5HT from these cells (Larsson, 1981). 5HT can be released either into the lumen of the intestine or the blood. Most of the 5HT released into the plasma will be metabolized by the liver and the endothelium but some will be taken up into platelets (Vanhoutte, 1983). A small, but probably physiologically significant proportion of 5HT in the gut is located within serotonergic neurons in the myenteric plexus. This has been demonstrated by immunocytochemical evidence (Dahlstrom and Ahlman, 1983) and the presence of tryptophan hydroxylase (Gershon et al., 1977). A trans-mucosal barrier has been demonstrated which prevents the high concentration of 5HT from the EC cells (mucosal side) from bathing the neurons (serosal side) (Gershon and Bursztajn, 1978; Gershon, 1982).

One of the earliest studies demonstrated that 5HT could be released from the intestine by intraluminal pressure (Bulbring and Crema, 1959), a finding supported by histochemical alterations in the cells themselves (Cole et al., 1961). Hypertonic glucose solutions can also stimulate release of 5HT (Drapanas et al., 1962) possibly by promoting the transport of the amine from the serosal side into the lumen (Narvanen, 1983). Luminal acidification is a stimulus to 5HT secretion. Acid at pH 5 applied to the mucosal surface will elicit 5HT release, which is not observed when the same stimulus is applied at the serosal surface. Alternatively, cholinergic and adrenergic antagonists could inhibit the acid stimulated release only when applied to the serosal surface (Kellum et al., 1984). In addition, there is evidence that 5HT can inhibit gastric acid secretion (Ormsbee and Fondacaro, 1985), suggesting a

possible feedback effect of 5HT on gastric acid secretion.

5HT has also been implicated in regulation of intestinal motility (Gershon, 1968; Ormsbee and Fondacaro, 1985). The large amounts of 5HT produced by the tumor in the carcinoid syndrome are associated with diarrhea, a symptom which can be ameliorated by administration of 5HT antagonists (Melmon et al., 1965) or synthesis inhibitors (Sjoerdsma et al., 1970). Whether 5HT exerts a stimulatory or inhibitory action on motility depends on which part of the intestine is being studied. The effect is primarily neuronal with no direct effect on smooth muscle (Ormsbee and Fondacaro, 1985). 5HT can inhibit the vagally evoked potentials during the descending inhibitory phase of the peristaltic reflex and also activate the intrinsic neurons which relax the smooth muscle (Gershon, 1982). Because of the diverse actions of 5HT on gut motility, it has been postulated that the diarrhea manifested during the carcinoid syndrome may also result from the action of 5HT on water and electrolyte transport. Kisloff and Moore (1976) found that 5HT administration resulted in an increase of both water and sodium secretion in the ileum and jejunum. This effect was inhibited by the addition of glucose to the perfusate.

An interesting relationship has been demonstrated between gut parasites and intestinal 5HT. Entamoeba histolytica, a protozoan parasite which causes diarrhea in humans was found to alter electrolyte transport on the serosal surface of the rabbit ileum and the rat colon. These effects were blocked by an analogue of 5HT and partially inhibited by an antibody to 5HT (McGowan et al., 1983). When rats were infected with Hymenolepis diminuta (Cestoda), 5HT significantly decreased glucose uptake compared to uninfected animals (Gruner and Mettrick, 1984).

5HT has been identified in both the endocrine (Falck and Hellmann, 1963; Ekholm et al., 1971) and the exocrine (Gershon and Ross, 1966) cells of the pancreas. Because of the site of localization, it was thought that 5HT may be involved in regulation of hormonal release. Early studies found that intracellular 5HT could be increased by uptake of either exogenous 5HT (Linstrom, 1981) or 5-hydroxytryptophan (5HTP) the precursor (Lundquist et al., 1971). Initially it was found that in mice, 5HTP inhibited sulphonylurea stimulated insulin release (Lundquist et al., 1971). Using microdissected islets from ob/ob mice, differential effects of 5HT and 5HTP on insulin release were found. 5HTP potentiated glucose-stimulated insulin release, while 5HT inhibited it (Linstrom and Sehlin, 1983a). It was suggested that the mechanism of 5HTP potentiation was mediated by the enzyme, amino acid decarboxylase (Linstrom and Sehlin, 1983b). Other groups have also found opposite effects of precursor and neurotransmitter. Pontiroli et al., (1978) demonstrated that in isolated islets and in pieces of pancreas, 5HT inhibited insulin release, but 5HTP had no effect. Similarly, Lebovitz and Feldman (1973) found 5HTP to be ineffective in contrast to 5HT in the golden hamster pancreas. In the same animal, when 5HTP was administered along with a monoamine oxidase inhibitor, a decrease in plasma insulin was reported (Bird et al., 1980). Ablation of serotonergic neurons in the rat pancreas had no effect on glucose-stimulated insulin release (Jansson et al., 1985). Differential effects are seen with low and high doses of glucose stimulation. In one study, 5HT stimulated insulin secretion at low concentrations of glucose (Galiardino et al., 1974). Administration of tryptophan was found to potentiate insulin releases at high doses of glucose in the ob/ob mouse islets (Lindstrom and Sehlin, 1986). In

isolated rat islets, tryptophan also stimulated insulin release only at a low glucose dose (Pontrioli et al., 1978). 5HT has also been implicated in regulation of glucagon release but the results are no easier to interpret. Matsumoto et al. (1984) found that in female syrian hamster islets, 5HT increased glucagon release, but Marco et al. (1977) found that release of the hormone was inhibited in mouse islets.

In the exocrine pancreas, 5HT is stored in zymogen granules of acinar cells and is thought to be secreted with amylase (Falardeau and Heisler, 1984). It has been hypothesized that 5HT may be modulating the rate of fluid secretion from the granule (Falardeau and Heisler, 1984) or be involved in it's formation and stability (Yu et al., 1984). As it has also been demonstrated that there is an efflux of 5HT from Beta cells in pancreatic islets from ob/ob mice (Gylfe, 1978), perhaps the transmitter is involved in storage, formation or release of granule contents.

It is very difficult to arrive at a conclusion concerning the biological role of 5HT in the pancreas. One problem is the considerable variation between species. The degree of innervation by noradrenergic fibers and the existence of other monoamines which may be effected by the various treatments may be partly responsible for the differential responses reported (Lebovitz and Feldman, 1973). In addition, it has been suggested that the action of 5HT on glucagon secretion may be mediated by a Beta receptor (Matsumoto et al., 1984). Tissue preparation will also influence the outcome of the experiment. Islets are more easily damaged than pancreatic pieces and cells located on the outer layer of the islet (Alpha cells) may be effected. Due to the paracrine actions of the other hormones, damage to one cell type may influence hormonal release. The differential effects of tryptophan, 5HTP and 5HT

lead one to suspect that different compartments of 5HT within the pancreas are altered. Administration of tryptophan, 5HTP and 5HTP in conjunction with a MAOI have different biochemical and behavioral effects in the whole animal (Modigh, 1972; Green et al., 1976). Not only does 5HTP bypass the rate limiting enzyme of 5HT synthesis but the enzyme which decarboxylates the compound to the neurotransmitter is not specific to serotonergic neurons. Therefore, adminstration of 5HTP may effect neuronal systems that would not be activted under physiological circumstances.

The pineal gland has the highest concentration of 5HT of any body tissue. Though it is located within the cranium, the pineal is technically not part of the brain as it is not protected by the blood-brain barrier. The pineal gland is considered a neuroendocrine organ, which responds primarily to photic stimuli (Cardinali, 1981). The major secretory product is melatonin but other compounds which are synthesized in the gland such as 5HT, N-acetyl serotonin (Ho et al., 1985) and 5-hydroxytryptophol (Feldstein et al., 1970) are biologically active as well.

The same biosynthetic pathway that is present in the brain for 5HT also exists in the pineal. Thus, tryptophan is taken up from the blood into the pinealocyte where it is converted to 5HTP and then 5HT. The 5HT can then be converted to N-acetyl serotonin, melatonin and other derivatives such as 5-hydroxytryptophol. A circadian rhythm has been demonstrated for all of the above mentioned compounds (Sugden, 1979; Young and Anderson, 1982) An inverse relationship betwen 5HT and melatonin synthesis has been demonstrated. 5HT levels drop during the night while melatonin increases (Young and Anderson, 1982). The decrease

in 5HT is thought to be due to the increase in melatonin synthesis caused by induction of N-acetyltransferase. As the magnitude of decline is in excess of that needed for melatonin synthesis, 5HT may be required for it's own biological functions (Cardinali, 1981). Increases in the activity of tryptophan hydroxylase are observed in the dark period and are abolished if the lights are left on (Sitaram and Lees, 1978). Pineal tryptophan rhythms are not correlated with serum total or free tryptophan indicating that the pineal can take up tryptophan against a concentration gradient (Sugden, 1979). Tryptophan administration (i.p. or oral) increases pineal tryptophan, 5HT and melatonin in the dark and the light (Sugden and Anderson, 1982).

1.3.6 The effect of acute dietary intake on tissue tryptophan and 5HT

In section 1.2.5, the effect of acute dietary intake on brain tryptophan was discussed. A handful of these studies have examined the effects on brain 5HT as well. This section will review dietary effects on brain 5HT and also the few experiments which have looked at the effect of diet on tryptophan and 5HT in tissues other than the brain. The effect of chronic dietary intake on brain levels of neurotransmitters presents a whole new set of variables. As all of the experimental work presented in the thesis has focused on the acute effects of dietary intake, the literature concerned with chronic effects will not be discussed.

As mentioned in the preceeding section, starvation alone influence brain amine levels. Twenty-four hour food deprivation increases plasma free tryptophan, the non-esterified fatty acids (NEFA) and brain tryptophan (Knott and Curzon, 1972). In one study, brain 5HT

was also increased (Curzon et al., 1972) though in two others no change was observed (Knott and Curzon, 1972; Perez-Cruet et al., 1972). Elevations in 5HIAA were uniformly reported. An increase in brain tryptophan of 44% led to a rise of 8% in 5HT and 38% in 5HIAA (Curzon et al. 1972). Various interpretations can be lent to this and the other studies which demonstrated large changes in 5HIAA levels. A lack of change or a small change in brain 5HT may be a result of an increase in 5HT turnover. The increased turnover might be due to an increase in the amount of 5HT released followed by an increase in metabolite, which would result in no net change in the level of brain 5HT. Alternatively, the increase in metabolite could be due to the metabolism of intraneuronal 5HT.

During starvation, it has been suggested that the liver maintains plasma tryptophan through hepatic output (Bloxam et al., 1974). Though portal venous plasma tryptophan is decreased during starvation in rats, hepatic venous levels are not different from fed animals. There is also a net uptake of tryptophan by extra-abdominal peripheral tissues (Bloxam et al., 1974). Other tissues, such as the heart and pancreas also exhibit elevated tissue tryptophan after starvation (Bender et al., 1975). In the perfused rat exocrine pancreas, an increase in the rate of neutral amino acid transport has been reported, but this was observed only after 72h of fasting (Mann et al., 1986) while the other experiments reported tissue changes after 24h. In one study liver tryptophan declined (Bloxam et al., 1974) but in the other an increase was observed (Bender et al., 1975). No change was observed in stomach tryptophan after fasting but 5HT declined by 31% upon refeeding of a standard rat chow. In the duodenum, an increase in tryptophan was observed between

the fed and fasted animal due to the increase in amino acid absorption after eating. In contrast 5HT declined in the duodenum (Biggio et al., 1977). It seems that in peripheral tissues, changes in 5HT can occur without parallel changes in tissue tryptophan.

The effect of carbohydrate on brain 5HT is consistent with changes observed in tryptophan. Carbohydrate has been shown to elevate brain 5HT in fasted rats (Fernstrom and Wurtman, 1971; Fernstrom and Wurtman, 1972;Fernstrom et al., 1975; Crandall and Fernstrom, 1980; Peters and Harper, 1987). The mechanism, as discussed in the previous section, is due to stimulation of the uptake of the branched chain amino acids into muscle by insulin, which is secreted upon carbohydrate ingestion, thereby lowering the concentration of the competing amino acids. In diabetic rats, the increase in brain 5HT does not occur due to impaired insulin release (Crandall and Fernstrom, 1980). The degree of change exhibited in brain 5HT after ingestion of a carbohydrate diet ranges from 18% (Fernstrom and Wurtman, 1971) to 26% (Fernstrom et al., 1975). Intragastric glucose administration increased brain 5HT by only 9% in normal rats (Crandall and Fernstrom, 1980). It does not seem likely that the smaller rise in brain 5HT by intragastric glucose as compared to consumption of a carbohydrate diet is due to the route of administration as comparable changes have been reported with intragastric administration of glucose (chapter 2). Intragastric glucose lowered duodenal 5HT but increased pancreatic levels, forty-five minutes after intubation (Brown, 1979).

If the competing amino acids minus tryptophan are added to the standard carbohydrate diet then a significant decline in brain 5HT and 5HIAA of 13% and 12% respectively, is observed (Fernstrom et al., 1975).

The addition of the non-neutral amino acids has no effect. Protein in combination with other dietary constituents has not been found to alter brain 5HT levels. One study which reported a decline in brain tryptophan did not measure 5HT (Glaeser et al., 1983), while others which did determine both compounds either found a decline in precursor only (Peters and Harper, 1987) or did not observe a change in either tryptophan or 5HT (Crandall and Fernstrom, 1980). Thus, an 18% casein diet did not alter brain tryptophan or 5HT but there was a significant 24% decline in 5HIAA (Crandall and Fernstrom, 1980). In rats fed diets ranging in protein content from 5 to 55%, no significant correlations were found between protein intake and brain 5HT or 5HIAA (Peters and Harper, 1987). Therefore, it seems that consumption of a pure carbohydrate meal will significantly increase brain 5HT in most circumstances while a protein containing meal will have a less consistent effect.

1.4.1 The role of 5-hydroxytryptamine in the acute regulation of food intake

1.4.2 5-hydroxytryptamine and regulation of food intake in animals

One question which is often asked with respect to the relationship between dietary intake and neurotransmitter synthesis is: Why should the brain be sensitive to precursor availability? An intuitively acceptable hypothesis has been that 5HT is involved in regulation of food intake. Anatomically, this is feasible as 5HT neurons project to the hypothalamus, an area of the brain essential to feeding. Consistent with is role as an inhibitory neurotransmitter in other behaviors such as pain (Roberts, 1984), aggression (Ogren et al., 1980; Malick, 1976) and sexual activity (Tagliamonte et al., 1969), increasing 5HT metabolism

also inhibits food intake.

Fenfluramine, a drug which increases the release of 5HT (Knapp and Mandell, 1976) is a potent anorectic in rats. It has been found to inhibit food intake in a variety of feeding paradigms such as food deprivation and tail pressure (Rowland et al., 1985). Evidence for the specificity of 5HT involvement in the food inhibiting effect was demonstrated by pharmacological treatments. Methysergide, a postsynaptic 5HT receptor blocker can antagonize the first hour of the appetite suppressant effect of fenfluramine (Blundell et al., 1973). Sim[illegible], an inhibitor of 5HT uptake can potentiate the anorectic effect of 5[illegible] (Goudie et al., 1976). In contrast, depletion of 5HT by inhibiting tryptophan hydroxylase (Breisch et al., 1976) or by intraventricular injection of the neurotoxin 5,7-dihydroxytryptamine will produce hyperphagia (Saller and Stricker, 1976), though there is some evidence to the contrary (Fuxe et al., 1975). In genereal, it is more difficult to stimulate food intake that to inhibit food intake, so treatments which deplete 5HT tend to give variable results as far as hyperphagia is concerned.

A more refined experimental technique, which reveals subtle differences in pharmacological and biochemical treatments, involves monitoring the micro and macro structure of feeding behavior. Length of time spent eating, number of eating bouts, rate of eating and intermeal intervals are some of the components which can be measured by recording feeding patterns in rats (Blundell, 1986). Using this method, fenfluramine slowed the rate of meal consumption and terminated the meal prematurely. In freely feeding rats, the drug reduced the size of the meal (Blundell et al., 1979). Shor-Posner et al. (1986) injected 5HT and

norfenluramine into the paraventricular nucleus of the hypothalamus and found similar effects, in that there was a marked reduction in meal size, duration and eating rate. Overall, there is agreement in the literature that 5HT acts as an inhibitory neurotransmitter on feeding behavior (Hoeble, 1977; Blundell, 1984; Leibowitz, 1986). More specifically, evidence suggests that it is influential in the induction of satiety (Blundell, 1979; Shor-Posner et al., 1986).

Though it is generally accepted that rats can regulate their food intake depending on their energy requirements, the concept of a separate mechanism for protein regulation only emerged when a decrease in food intake was demonstrated after administration of an amino acid imbalanced diet (Harper et al., 1970). More recently, this concept has been substantiated by showing that weanling rats can increase or decrease their protein intake depending on the quantity or quality of protein available (Musten et al., 1974). Because 5HT is synthesized from tryptophan, it was a plausible neurochemical candidate as a regulator of protein intake. A biochemical-behavioral feedback loop was hypothesized in which dietary intake could alter brain levels of 5HT, which in turn would subsequently alter protein selection (Anderson, 1979). Alternatively, other groups suggested that 5HT was regulating carbohydrate intake (Wurtman and Wurtman, 1979) or that the transmitter was not involved in macronutrient selection at all (Peters and Harper,1984).

The theories relating 5HT to control of protein and carbohydrate can, in some ways be regarded as different aspects of the same overall mechanism. Thus, protein intake will tend to decrease 5HT, and lowered brain 5HT will then inhibit selection of protein (relative to

carbohydrate). On the other hand, carbohydrate raises brain 5HT and increased 5HT function will inhibit selection of carbohydrate (relative to protein). Thus, the variable that is being influenced may be the relative quantities of protein and carbohydrate that are ingested. While this is an interesting hypothesis, the evidence for it is far from complete. As discussed above, the evidence that protein and carbohydrate influence brain 5HT is not entirely consistent, while the evidence that 5HT influences macronutrient selection is derived from non-physiological exeriments. Drugs treatments have tended to demonstrate consistent effects of 5HT manipulations on macronutrient selection. It is in these experiments that compensatory mechanisms in food selection are manifested, seemingly as a result of changes in brain 5HT. Thus, both fenfluramine and fluoxetine, two drugs which increase the amount of 5HT in the synaptic cleft, were found to increase protein intake as a percent of total calories in rats trained to consume all their food within an eight hour dark period (Wurtman and Wurtman, 1977). In freely-feeding rats a similar effect was demonstrated. However, in a different experiment fenfluramine reduced total food intake while maintaining the percent of energy derived from protein (Blundell et al., 1979). Wurtman and Wurtman (1979) showed that when the protein content of the diet was held constant, rats could regulate their carbohydrate intake independent of taste. They found that fenfluramine and MK-212, another 5HT anorectic agent, significantly decreased the amount of carbohydrate rats selected as compared to control animals. From these experiments, it was proposed that 5HT was involved in regulating the consumption of foods which would effect it's own synthesis, i.e. carbohydrate.

Ashley et al.(1979) examined the separate effects of

para-chlorophenylalanine and 5,7 dihydroxytryptamine on food selection in weanling rats. In these experiments, animals were maintained on a self-selection paradigm over an extended period of time either before or after treatment. Both treatments, which significantly decreased brain 5HT levels, resulted in a specific decline in protein selection. Lesions in the raphe exhibited a similar effect. Central injection of 5HT or norfenfluramine resulted in a selective decline in carbohydrate ingestion. Animals in this experiment were presented with isocaloric diets containing either casein or dextrin (Shor-Posner et al., 1986).

Tryptophan administration has been shown to have no effect (Peters et al., 1984) or decrease food intake in rats (Latham and Blundell, 1979; Morris et al., 1987). With higher doses, carbohydrate selection is inhibited as well as total calorie intake (Morris et al.,1987). A component of the effect observed may be peripherally mediated, as when valine was co-administered with tryptophan the decline in food intake persisted. In fact, valine alone significantly reduced total food intake (Morris et al., 1987). A peripheral role of 5HT in the regulation of food intake has been postulated in a number of studies. Systemic injection of 5HT, which cannot cross the blood-brain barrier, significantly reduces food intake in food deprived animals (Pollock and Rowland, 1982; Fletcher and Burton, 1984). The anorectic effect of peripheral 5HT is enhanced by sub-diaphragmatic vagotomy (Fletcher and Burton, 1985). The mechanism by which vagotomy enhances 5HT-induced anorexia is unclear. Though vagotomy has been shown to reduce gastric emptying, a dissociation was demonstrated between reduced gastric clearance and the reduction in food intake by the admnistration of methysergide. The 5HT antagonist blocked the anorectic action of 5HT in

vagotomized rats but did not reverse the reduced gastric clearance induced by 5HT. An interaction between vagal input and 5HT in the enterchromaffin cells was postulated (Fletcher and Burton, 1985).

The physiological regulation of food selection on a meal to meal basis, without pharmacological manipulations, has been examined in few experiments. Li and Anderson (1982) administred either a 45% or 0% protein diet and thirty minutes later allowed the rats to select from diets cotaining 10% or 60% casein. A decline in protein and an increase in carboydrate intake was exhibited. If the protein concentration of the premeal was increased to 70%, total food intake was decreased as well as protein intake. Wurtman et al. (1983) found that rats given a carbohydrate, as opposed to a mixed nutrient diet, selected less carbohydrate from a choice of 25 and 75% dextrin diets compared to the control animals. Although these studies which show tht rats are able to adjust their macronutrient content are interesting, they provide no evidence that these effects are mediated by 5HT.

The studies discussed above are consistent with the data that 5HT is involved in regulating macronutrient selection on a meal to meal basis even if the evidence is incomplete. Thus, lowering brain 5HT will result in a propensity of the animal to select a meal which would cause a subsequent increase in 5HT metabolism, either by increasing carbohydrate or decreasing protein intake. Alternatively, increasing brain 5HT inhibits carbohydrate or increases protein selection. Unfortunately, one disadvantage of the self-selection paradigm is that in most circumstances animals are offered a choice of either a high or low macronutrient diet. If for example, a high carbohydrate diet is selected, one does not know if this is due to a carbohydrate preference or a protein inhibition.

Possibly, 5HT is involved in the regulation of the proportion of protein to carbohydrate rather that one macronutrient in particular. Theall et al. (1984) demonstrated that rats would maintain the proportion of protein to carbohydrate when allowed to select from isocaloric diets differing in carbohydrate content.

It is necessary to emphasize the importance of experimental design in feeding studies. As illustrated in the self-selection paradigm, and by the use of structural temporal analysis, information concerning the finer components of food intake can only be drawn out if the design of the experiment is sensitive to these components. An important but rarely regarded aspect of food intake is the sensory quality of the foods offered. Rats will alter dietary intake depending on whether the diet is presented in the form of a gel, powder or granulated form (Blundell, 1983). Lack of consistency in the form of diet offered, is most likely one cause of the many discrepancies observed in the dietary studies. An additional factor is the satiating capacity of the different macronutrients. Isocaloric quantities of protein are more satiating than the same amount of carbohydrate (Blundell and Hill, 1987a; Jen et al., 1985).

In conclusion, this review has presented the existing experimental data for the role of 5HT in the regulation of acute food intake and macronutrient selection. As the focus of the thesis is the effect of acute dietary intake on 5HT metabolism, function and behavior, chronic dietary studies have not been examined. Nor have I presented the vast literature on other aspects of the regulation of food intake. Food intake regulation involves neurochemical, biochemical and hormonal factors. Compounds in each of these categories have been proposed as

regulators of food intake (Anderson, 1979; Wurtman et al., 1981; Mayer, 1955; Le Magnen; 1984; Woods et al., 1981; Woods et al., 1985). It would be naive to think that a behavior as complex as food intake would be under the control of only one chemical component

1.4.3 5HT and regulation of food intake in humans

Regulation of food intake in humans encompasses a wider variety of behavioral responses to physiological and environmental cues than laboratory animals possess or are permitted to express. In addition to the components discussed above such as palatability, satiety and selection availability, psychological and cultural factors play an important role in food selection in humans. The wide range of response observed in humans is a reflection of these components. Thus, designing experiments which can both overcome the large variability and be sensitive enough to monitor subtle changes in behavior is very difficult. The number of studies which have actually examined the role of 5HT in the regulation of acute food intake in humans is very small, therefore a few studies which are clinically relevant to the subject will be included though they examined food intake over a longer period of time.

Blundell et al.(1979) examined the effect of fenfluramine on food intake and macronutrient selection in 12 subjects (6 male, 6 female) over 24 hours, using a similar design as in his animal experiments. After administration of either drug or placebo, subjects were brought into a common room and allowed to select their subsequent lunch, dinner and breakfast from isocaloric portions of a variety of sandwiches. Videocaping occurred behind a one-way screen and behavior was later analyzed for the duration of eating, rate of ingestion, inter-mouthful interval etc. Fenfluramine significantly reduced kilocalorie intake by 26% while maintaining the proportion of protein in the meal. As was observed in the

animal studies, the drug slowed the rate of eating.

In another study, fenfluramine was administered to 11 normal subjects who claimed to have a propensity for carbohydrate snacking (Wurtman and Wurtman, 1981). The theoretical basis of this experiment is derived from the hypothesis that "carbohydrate cravers" have abnormal central 5HT metabolism, possibly due to lowered brain 5HT or a defective feedback inhibition which results in a continual craving for carbohydrate. Therefore, eight day food records were obtained in order to determine the pattern of snacking and the type of snack preferred. Subjects were administered the drug one hour prior to their predetermined snacking time. If that time coincided with meal time, then they were permitted to eat the meal. Food intake was recorded by the subjects themselves on special sheets. Fenfluramine significantly reduced the consumption of carbohydrate snack food over a five day period in the group as a whole, but individual responses were highly variable. A pronounced effect was observed in six of the subjects while four were virtually unaffected. Six of the subjects consumed sufficient mealtime non-snack carbohydrate foods to allow comparison with placebo. It was found that all six surpressed carbohvdrate consumption. As the results of this experiment are based on the subjects' personal recording consistency, and food intake records are known not to be reliable indexes of nutritional intake, results of this experiment cannot be interpreted with much confidence.

L-tryptophan has been used in a number of studies to increase brain 5HT and to observe the effect on food intake in normal and obese subjects. In the study by Wurtman and Wurtman (1981) just described, two grams of tryptophan per day administered in one dose were given to carbohydrate snackers, one hour prior to the designated snacking time. No statistically significant differences were found between experimental and placebo groups.

Upon individual examination, four subjects reduced their consumption of carbohydrate snack foods while four increased their intake. Three were not affected. In a double-blind crossover study (Hrboticky et al.,1985), subjects were given tryptophan after an overnight fast and a standardized breakfast, upon which they were allowed to leave the laboratory. They were then required to return at lunch time and allowed to select from a variety of nine foods composed of luncheon meats, cheeses, cookies and bread. Food intake was measured by difference. Two grams of tryptophan was found to be effective in reducing energy intake. In one trial, energy intake was reduced by 13% and in the second, a 19% reduction was observed. One gram of tryptophan had no significant effect while three grams did not cause a further reduction in food intake compared to the two gram dose. A nutritional reduction in the protein/carbohydrate ratio was observed after two grams but this result was not repeated in the second experiment. After both the two and three gram doses of tryptophan, subjects reported increased dizziness and faintness. It is possible that the side effects of tryptophan ingestion interfered with food intake, though the authors suggest that the anorexigenic effect can be separated from the psychotropic effecs due the magnitude of response. While changes in mood were dose-related, the suppression in food intake was the same for two and three grams.

Theoretically, tryptophan administered simultaneously with a high carbohydrate meal should potentiate the increase in brain serotonin and therefore accentuate any responses in food intake. In fact, Ashley et al. (1985) demonstrated that a carbohydrate diet plus tryptophan significantly elevated the tryptophan ratio compared to the carbohydrate diet alone. Blundell and Hill (1987b) administered one gram of tryptophan in the form of a chocolate bar, in conjunction with either a high protein or high carbohydrate lunch.

In total four different diets were tested; protein alone, protein plus tryptophan, carbohydrate alone, carbohydrate plus tryptophan. They then offered a tea-time meal from which subjects were allowed to select foods they had previously rated as highly desirable. The high protein lunch led to a 16% decrease in kilocalories selected at tea-time compared to the high carbohydrate lunch, while the addition of tryptophan had no significant effect on food intake. The protein lunches suppressed protein consumption by 27% compared to the isocaloric high carbohydrate lunches. When the high protein lunch was combined with tryptophan, there was an significant decrease in carbohydrate intake (15%). No differences were observed between carbohydrate and carbohydrate in combination with tryptophan. Several important points can be drawn from the results of this experiment. In humans, like animals, protein exhibits a greater satiating effect than isocaloric amounts of carbohydrate. Also, previous studies have not found significant effects with a dose of tryptophan as low as one gram. The combination of tryptophan with a high protein diet magnified the effect of tryptophan alone. Interestingly, the effect was not in the direction that would have been hypothesized. A potentiating effect of carbohydrate plus tryptophan was not observed, possibly because 5HT synthesis was relatively close to a saturation point. However, protein in combination with tryptophan demonstrated a specific and significant suppression of carbohydrate intake, suggesting that brain 5HT levels were elevated from the treatment. One possible interpretation of the phenomenon is that ingestion of protein lowered the baseline levels of the tryptophan ratio thereby allowing a greater response to the administration of tryptophan.

The connection between carbohydrate regulation and 5HT metabolism has led to a number of studies designed to look at the relationship between 5HT and obesity. As discussed above, it is thought that certain forms of

obesity associated with the excessive intake of carbohydrate may be due to some form of abnormal 5HT metabolism (Ashley et al., 1985). Though fenfluramine consistently reduces food intake in humans, a reduction in carbohydrate intake was demonstrated as well in obese subjects (Wurtman and Wurtman, 1984). Strain et al. (1984) gave one gram of tryptophan along with 10 grams of carbohydrate, three times a day to obese subjects for six weeks. Despite the significantly elevated plasma tryptophan levels, treated subjects did not lose more weight than those on placebo. The concentration of the competing amino acids were not measured but some groups have demonstrated a blunted decline in the plasma branched-chain amino acids after insulin release in obese subjects (Felig et al., 1969; Caballero, 1987). If carbohydrate ingestion does not elevate the plasma tryptophan ratio, therefore brain 5HT will not increase. In fact, even after tryptophan administration, the ratio does not increase to the same extent as exhibited in lean subjects (Caballero, 1987).

The experiments discussed above are relevant to the experimental work in the following chapters from two different perspectives. Firstly, monitoring changes in behavior after tryptophan or dietary intake can offer insight into mechanisms regulating 5HT metabolism. More importantly, in humans, alterations in behavior are one of the few ways of assessing the role of functional 5HT. Secondly, the clinical implications in understanding the role of 5HT in the regulation of food intake are vast. The high prevalence of eating disorders, obesity at one end of the spectrum to anorexia at the other, emphasizes the necessity in determining the factors and mechanisms involved in the regulation of this complex behavior.

1.5.1 Conclusion

The preceding sections attempted to present a review of the literature

and highlight some of the important questions that have been left unanswered in the experimental work to date. In addition, the many variables and the importance of experimental design have been emphasized. One aspect which was not discussed, but was briefly mentioned in the introduction was the behavioral effect of food in humans.

The scientific evidence supporting or refuting the behavioral effects of food has only recently begun to accumulate. An interesting finding stemming from the ergonomic literature suggests that the phenomenon known as "post-lunch dip" may be related to food intake. The post-lunch dip, which occurs 1-2 hours after ingestion of the midday meal is generally associated with a decline in alertness and efficiency. Frequency of errors and decreases in reaction time in a variety of motor tasks have been reported (Craig, 1986). Though diurnal variation may play a role, Craig et al. (1981) found that the consumption of a meal at lunch time impaired the subjects performance on a perceptual discrimination task.

Other lines of evidence stem from the effect of fasting on cognitive function (Pollitt et al., 1983) and from the effects of food ingestion on mood (Spring et al., 1983; Lieberman et al., 1983; Lieberman et al., 1986). Generally, carbohydrate has been found to exert a sedative effect (Spring et al., 1983; Lieberman et al., 1986). In most cases, psychological effects were thought to be mediated by changes in the tryptophan ratio due to macronutrient ingestion. If this were the case, then ingestion of physiological amounts of protein or carbohydrate should alter 5HT metabolism, function and finally behavior.

The following six chapters will contain experimental work designed ultimately to determine the effect of protein and carbohydrate on 5HT metabolism and function. Half of the studies presented were performed on the Sprague-Dawley rat and the other on consenting human subjects. In both

models, we were interested in answering specific questions. These were: Can protein and carbohydrate alter 5HT metabolism? and Can protein and carbohydrate alter 5HT function? Chapter 2 examines the effect of the two macronutrients on tryptophan and 5HT metabolism in the brain and three peripheral tissues in the rat. Chapter 3 proposes and validates a new methodology for the determination of functional 5HT in the rat. Using the new technique, chapter 4 examines the effect of protein and carbohydrate on functional 5HT. Following parallel lines in the human work, to address the question of the behavioral effects of functional 5HT, we manipulated dietary tryptophan and examined the effect on food selection (chapter 5). Chapter 6 approaches the question of 5HT metabolism and function by determining the effect of protein and carbohydrate ingestion on the plasma tryptophan ratio and food selection. Finally, chapter 7 demonstrates the effect of macronutrient ingestion on 5HT metabolism by the measurement of tryptophan and 5HIAA in human CSF.

1.6.1 References

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PREFACE TO CHAPTER TWO

The objective of the experiments in this chapter was to determine if administration of the macronutrients, protein and carbohydrate, could influence tryptophan and 5HT concentrations in different organs of the rat. Earlier work had demonstrated that carbohydrate could raise tryptophan and 5HT levels in the brain. In some studies, protein was shown to lower brain tryptophan but no lowering of brain 5HT had been shown. These studies had used either a mixed diet containing both protein and carbohydrate or a mixture of amino acids. In the present experiments, both macronutrients were administered in a purified form. As well, the compounds were given intragastrically, a method which avoids the confounding aspect of sensory influences.

Despite evidence implicating the role of peripheral 5HT in the regulation of food intake, the effect of diet on 5HT in peripheral tissues had not been examined. This experiment was designed to determine if tryptophan availability could influence 5HT levels in tissues which lie outside the blood-brain barrier. Three tissues, known to contain 5HT, were examined: the pineal, which lies within the cranium but outside the blood-brain barrier, would not be expected to be under the same regulatory mechanisms as the brain, since competition between the other large neutral amino acids should not take place: the intestine which contains large amounts of 5HT and many peptides thought to be involved in the regulation of food intake and lastly, the pancreas because of its crucial endocrine role, which is an integral part of the mechanism regulating brain 5HT levels. Therefore, the effect of protein and carbohydrate on tryptophan and 5HT were examined in these tissues to determine if dietary manipulations could alter 5HT metabolism.

Chapter 2

EFFECTS OF CARBOHYDRATE AND PROTEIN ADMINISTRATION ON RAT TRYPTOPHAN AND 5-HYDROXYTRYPTAMINE: DIFFERENTIAL EFFECTS ON THE BRAIN, INTESTINE, PINEAL AND PANCREAS

ABSTRACT

We compared the acute effects of intragastric administration of protein and carbohydrate on tryptophan and 5-hydroxytryptamine (5HT) in rat brain, pineal, intestine and pancreas. Protein decreased and carbohydrate increased brain indoles relative to water-infused controls. These effects were due to competition between the large neutral amino acids for entry into brain. This competition does not exist in the pineal. The macronutrients had no effect on pineal tryptophan metabolism. In the intestine, protein resulted in higher tryptophan levels as compared to controls, due to absorption of tryptophan in protein. However intestinal 5HT levels were influenced by factors other than precursor availability. Pancreatic indoles were affected in a similar manner to the brain indoles. Competition between the large neutral amino acids for entry into the pancreas was also indicated by the finding that valine administration lowered brain and pancreatic tryptophan, but not the levels in the intestine and pineal. It remains to be seen whether the decrease in pancreatic 5HT after a protein meal and the increase after carbohydrate modulate release of insulin and glucagon.

INTRODUCTION

Tryptophan hydroxylase, the rate limiting enzyme in the synthesis of 5-hydroxytryptamine (5HT), is not saturated with tryptophan under normal circumstances. Thus, changes in brain tryptophan levels can influence the rate of synthesis, and the content of brain 5HT (Wurtman et al., 1981; Young 1983). Brain tryptophan can be influenced acutely by intake of food. However, the changes in brain tryptophan are not in the direction that might initially be expected as protein, which contains tryptophan, lowers brain tryptophan, while carbohydrate increases it. This is because of competition between all the large neutral amino acids for entry into brain across the blood-brain barrier. A protein meal raises plasma tryptophan, but because tryptophan is the least abundant amino acid in most proteins, the concentration of the other large neutral amino acids in plasma rises even more. The increased competition for transport into brain can cause brain tryptophan to fall according to some researchers (Perez-Cruet et al., 1972; Glaeser et al., 1983) but others found no change (Fernstrom and Faller, 1978). When carbohydrate is ingested, the insulin that is released causes the branched chain amino acids to be taken up into muscle. Consequently, their plasma levels fall, there is diminished competition for entry into brain, and brain tryptophan and 5HT increase (Ferstrom and Wurtman, 1971) or are not effected (Glaeser et al., 1983).

Only 1% of total body 5HT is located in the brain, the rest being in the peripheral tissues and blood. Considering the large body of literature on the relationship between dietary intake and 5HT, few reports have been directed towards the effect of food intake on 5HT in tissues which lie outside the blood brain barrier, despite evidence for the involvement of peripheral 5HT in regulation of food intake (Pollock

and Rowland, 1981). The objective of this study was to compare the effects of intragastric administration of protein and carbohydrate on tryptophan and 5HT in the brain, intestine, pineal and pancreas.

The highest concentration of 5HT within the cranium is found in the pineal. The pineal is technically not part of the brain but of the peripheral nervous system and lies outside the blood brain barrier. Administration of tryptophan increases pineal 5HT and 5-hydroxyindoleacetic acid (5HIAA) (Young and Anderson 1974). It is not known whether there is competition between the large neutral amino acids for entry into pineal, or whether pineal tryptophan is affected by food intake.

The largest source of peripheral 5HT is in the intestine where it is found in the enterochromaffin cells, and also within neurons in the myenteric plexus. The role of 5HT in the intestine has not been fully elucidated although it has been implicated in the transport of water and electrolytes (Kisloff and Moore 1976), gut motility (Gershon 1968) and regulation of food intake (Pollock and Rowland 1981). Differences in 5HT levels in the duodenum of fed and fasted rats were reported by Biggio et al. (1977) suggesting that duodenal 5HT could be altered by dietary intake.

5HT is also present in both the alpha and beta cells of the pancreas (Ekholm et al. 1971). The physiological role of 5HT in the pancreas is controversial due to species differences and a lack of correlation between in vitro and in vivo studies. 5HT has been reported both to increase (Gagliardino et al. 1974) and to decrease insulin release (Lebovitz and Feldman 1973). In addition, it may be involved in modulation of glucagon secretion (Matsumoto et al. 1984). Brown (1979)

reported an increase in pancreatic 5HT after an oral glucose load was administered to rats which had been maintained on diets containing varying amounts of protein.

MATERIALS AND METHODS

Tryptophan as the free amino acid, valine and 5HT as the creatinine sulfate complex were purchased from Sigma Chemical Co., St. Louis, Mo.. Male, Sprague-Dawley rats, obtained from Charles River Canada, Inc. St. Constant, Quebec and weighing 190-210g, were maintained on a 12hr-12hr light-dark cycle. For all experiments there were eight animals per group and animals were randomly assigned to different groups. The rats were fasted overnight and treatments were initiated in the morning, 3 hrs after the onset of the light period. To look at the effect of protein administration on tissue indole levels, 4 mls of a 25% w/v solution of albumin was given by intubation. An identical volume of water acted as the control. In one experiment brains, intestines and pineals were collected, while in a separate experiment only the brains and pancreas were removed. The results for the brain were similar in the two experiments and Table 1 includes data on the brains from the first experiment only. Animals were decapitated two hours after intubation. To examine the effects of protein on plasma tryptophan an additional experiment with the same protein treatment was carried out, with blood being withdrawn by syringe from both the portal vein and the heart, 0.5 hr and 1.0 hr after intubation. Animals were anaesthetized with nembutal for this procedure.

The carbohydrate treatment consisted of 4 mls of a 50% w/v glucose solution, which was administered by intubation, with water as the control. As most meals generally contain twice the amount of carbohydrate

as protein, we followed these proportions and administered two times the quantity of carbohydrate as protein. Animals were killed 2.0 hr after intubation. Different groups of rats were used to take pancreatic tissue and all the other tissue. A separate time course experiment was carried out with half the animals killed at 0.5 hr and the other half killed at 1.0 hr after glucose intubation. Valine was given intragastrically at a dose of 400 mg/kg in 4ml of water. Tryptophan (100 mg/kg, 10 ml/kg) was injected intraperitoneally in isotonic saline. Due to the low solubility of tryptophan, 10 ml/kg was the minimum volume needed to dissolve the amino acid. Tryptophan was given intraperitoneally rather than intragastrically because the purpose of the experiment was to see if pancreatic 5HT was susceptible to alterations of tryptophan availability. Absorption is faster after intraperitoneal injection than after intragastric administration, and therefore tryptophan availability would be greater with this route of administration. In both these experiments the rats were decapitated 2 hrs after treatment. After decapitation brains, pineals and pancreases were removed and frozen immediately at -70°. The intestine was removed and rinsed inside and outside with saline before freezing. Fatty and connective tissue were removed from both the intestine and the pancreas after freezing at -70°.

For the brain, intestine and pancreas a 25% homogenate was made using 0.4M perchloric acid and a Polytron Homogenizer. Pineals were sonicated in 0.2ml of 0.1M perchloric acid. After centrifugation an aliquot of the supernatant was injected directly into a high performance liquid chromatograph. For the brain and pineal, tryptophan and 5HT were determined by the method of Anderson et al. (1981) which employs a Waters C18 u-Bondapak reverse-phase column and electrochemical detection. For

the intestine and pancreas this method was not adequate as the ratio of response on electrochemical and fluorometric detectors was different for the standards and samples. However, different buffer systems were found which gave the same response ratio. For intestine the buffer was 0.01M sodium acetate, pH 3.5 with 3% acetonitrile and for the pancreas 0.01M sodium acetate, 0.1mM sodium octyl sulfate, pH 4.25 with 12% methanol. Recovery of internal standards, determined by adding known amounts of 5HT to aliquots of homogenate, was 70% for the intestine and 85% for the pancreas. Tryptophan in plasma was measured by the fluorometric method of Denckla and Dewey (1967).

Statistical analysis of differences between two groups was performed using Students' t test.

RESULTS

Table 1 shows the differential effects of protein and carbohydrate on the four different tissues, two hours after administration of the meals. Carbohydrate increased brain indoles, while protein lowered them with respect to water-intubated animals. Unlike the brain, pineal levels of the indoles remained unaltered with either the protein or the carbohydrate treatment. The unexpected lack of effect of protein on pineal tryptophan led us to examine plasma levels. Tryptophan was unaltered in portal or cardiac blood after protein administration, although there was a trend towards an increase in portal values relative to controls (Table 2). In the intestine tryptophan was higher after protein administration, while carbohydrate lowered tryptophan levels (Table 1). Neither treatment had any effect on intestinal 5HT. Changes in the pancreas were similar to those seen in the brain. Pancreatic tryptophan and serotonin were lowered by protein. Glucose administration

resulted in higher pancreatic tryptophan but 5HT levels remained unaltered in comparison to controls.

To determine whether 5HT in the intestine and pancreas could be altered by changes in tryptophan levels, we looked at indoles in these organs after tryptophan administration (Table 3). The large rise in tryptophan in both tissues (55% in the intestine and 92% in the pancreas) resulted in elevated 5HT levels as compared to the saline injected group.

Because carbohydrate did not elevate pancreatic 5HT even though tryptophan was increased we decided to look at the effect of glucose at two earlier time points, 0.5hr and 1.0hr after treatment (Table 4). Pancreatic tryptophan was higher at 0.5hr and remained elevated at the later time point. A significantly higher pancreatic 5HT level was observed one hour after glucose administration. Tryptophan in the intestine was not changed at either 0.5hr or 1.0hr but 5HT levels were significantly elevated at 1.0hr with respect to untreated animals.

The experiment with protein suggested that tryptophan was competing with the other large neutral amino acids for entry into brain and pancreas but not pineal and intestine. To confirm this, valine, one of the competing amino acids, was given (Table 5). As expected, no change relative to controls was observed in either the pineal or the intestine, but valine treatment caused a significant lowering of pancreatic tryptophan.

DISCUSSION

Our results show that acute intragastric administration of the macronutrients, protein and carbohydrate, has differential effects on tryptophan metabolism in different tissues containing 5HT and that these effects are specific and unique to each tissue. Brain tryptophan and 5HT

were higher after carbohydrate administration, confirming other studies (Fernstrom and Wurtman, 1971; Fernstrom and Faller, 1978). Intragastric protein significantly lowered brain tryptophan and 5HT as compared to fasted controls. Both tryptophan (Glaeser et al, 1983) and 5-hydroxyindoleacetic acid, the metabolite of 5HT (Perez-Cruet et al., 1972) are lowered after dietary protein, although to our knowledge alteration in brain 5HT have not been reported. Conflicting data has also been presented in which brain tryptophan (Fernstrom and Faller, 1978) and brain 5HT (Peters and Harper, 1987) were unaffected by protein administration. Obviously, there are factors which can influence the relationship between protein ingestion and brain indole levels, which remain to be determined.

The pineal was the only tissue examined in which neither dietary component altered tryptophan or 5HT. As the pineal lies outside the blood-brain barrier, levels of tryptophan in the pineal should directly reflect plasma tryptophan, since there will be no competition for uptake from the other large neutral amino acids. This conclusion is confirmed by our finding that valine administration had no effect on pineal tryptophan and 5HT even though it lowered their levels in the brain (Table 5). Similarly, protein failed to lower pineal tryptophan although it lowered brain tryptophan (Table 1). After protein administration there was no change in either plasma or pineal tryptophan (Table 2). Plasma tryptophan at either 0.5 or 1 hour after protein infusion did not differ from water-infused rats. This agrees with the data of Crandall and Fernstrom (1983) who found no change in plasma tryptophan after rats were fed a meal containing 18% casein, though Glaeser et al. (1983) found significant increases relative to fasted animals. In humans, the

response of plasma tryptophan is more consistent and a protein meal elevates plasma tryptophan by 20% (Ashley et al. 1982). Studies in the rat indicate that tryptophan administration can increase pineal 5HT and melatonin (Young and Anderson 1982). However, the effect is relatively small and it is unlikely that a 20% increase in plasma tryptophan would have any appreciable effect on human pineal indole metabolism. Thus, it seems that as far as 5HT metabolism is concerned, the pineal, unlike the brain, is not sensitive to acute dietary manipulation.

Intestinal tryptophan appears to be influenced by the diet. The increase of tryptophan in this tissue after protein administration (Table 1) confirms a previous report, where similar changes were seen in duodenal tryptophan in rats fed a meal (Biggio et al. 1977). The increase could be due to the amino acid being taken up into cells either from the circulation or directly from the gut lumen. As no increase in plasma tryptophan was observed after protein ingestion, it seems likely that the rise in intestinal tryptophan is due to an increased availability of amino acids in the lumen after hydrolysis of the protein in the gut. Under these circumstances competition between the different amino acids for transport from the lumen into the intestine does not seem to play a significant role. Competition is also absent between blood and gut, as valine failed to lower intestinal tryptophan (Table 5). This is not surprising considering that the Km of amino acid transport into the intestine is much higher than the plasma amino acid levels (Pardridge and Oldendorf 1977) and that competition does not take place unless the plasma levels are of the same order of magnitude as the Km.

The effects of glucose on intestinal indole levels are inconsistent

and difficult to interpret. Different results are obtained depending on the length of time after treatment. A small but significant decline in intestinal tryptophan in the glucose intubated animals (Table 1) is a new and somewhat surprising finding. A decline of tryptophan of this magnitude (21%) would not necessarily be large enough to cause a significant decline in 5HT, which we found unchanged at the two hour time point (Table 1). Our data after tryptophan loading (Table 3) indicate that intestinal 5HT can be influenced by precursor availability. However, changes in intestinal tryptophan do not always influence intestinal 5HT. This is illustrated by the effect of glucose 1.0hr after administration (Table 4). Here we see an increase in 5HT, with no change in tryptophan relative to the control group. A disassociation between precursor and amine was reported by Biggio et al. (1977) who found an increase in intestinal tryptophan concomitant with a decrease in 5HT, three hours after chow feeding in rats. Lowered intestinal 5HT levels were also seen in rats 45 minutes after glucose administration (Brown 1979). This effect could be due in part to lowered tryptophan, though after chow, which contains protein, it is unlikely that the decline in 5HT is due to change in the precursor. One mechanism that might be involved is increased intraluminal pressure, which has been found to decrease gut 5HT (Bulbring and Lin 1958).

The effects of glucose, protein and valine on pancreatic tryptophan (Tables 1 & 5) were similar to those for brain tryptophan, i.e., protein and valine decreased the amino acid while carbohydrate raised it with respect to control. These unexpected results suggest that there is competition between the large neutral amino acids for entry into the pancreas. Until now it had been thought that this phenomenon was limited

to the brain (Pardridge and Oldendorf 1977). The blood-brain barrier to large neutral amino acids occurs at the level of the capillaries. The "blood-pancreas" barrier could also occur at the level of the capillaries (although we are not aware of any histological or cytological evidence for this) or at the cell membrane. Although the hypothesis that the specificity of competition in the pancreas is the same as in the brain (i.e., between the large neutral amino acids), it is also possible that the pancreatic transport system has a differential specificity. Extensive cross-inhibition studies will be necessary to determine the situation.

Before considering the effect of dietary intake on pancreatic 5HT it is necessary to discuss a little of what is known about 5HT in this organ. Monoamines were first identified in the pancreatic islets using a formaldehyde-induced histochemical fluoresence technique (Falck and Hellmann 1963). Much of the subsequent work has also involved histochemical methods and there are only a few papers which reported on the quantitation of 5HT. Bender (1974) using unspecified methodology, found 0.46-0.64 ug/g, while Brown (1979) found 0.27-0.35 ug/g using a spectrofluorometric method. Both these studies were in the rat. Using a radioenzymatic assay, Bird et al. (1980) found 0.704±0.331 (mean±SD) ug/g in hamster pancreas, even though the histochemical fluorescence technique was unable to demonstrate the presence of 5HT in hamster islets (Cegrell 1968). The levels of 5HT we found in rat pancreas are an order of magnitude less than those above. In our initial attempts to quantitate indoles in the pancreas using reverse-phase HPLC and electrochemical detection we found somewhat higher values. However, the use of fluorometric and electrochemical detectors in series indicated that other

compounds were co-eluting with the 5HT. After trying different chromatographic conditions we developed an assay in which quantitation was the same using electrochemical and fluorometric detectors. As it i extremely unlikely that an interfering compound would have identical chromatographic, fluorometric and electrochemical properties to 5HT, we have considerable confidence in the validity of this method. On the other hand, the spectrofluorometric method used by Brown (1979) and the radioenzymatic method used by Bird et al (1980) relied on simple extractions in their separation steps and, thus, may have lacked specificity. Our results showed some variability between control values in different experiments, but this type of variability is certainly know to occur for brain 5HT (Valzelli et al. 1977) and for pancreatic tryptophan (Bender et al. 1975) and, therefore, is not surprising.

The large decrease in pancreatic tryptophan after protein ingestion is accompanied by a decline in 5HT (Table 1) but the smaller fall in tryptophan after valine administration does not result in any significant change (Table 5). Similarly, the large increase in pancreatic tryptophan after tryptophan is given (Table 3) results in a significant increase in 5HT. However, the smaller increase in pancreatic tryptophan that occurred after carbohydrate feeding increased 5HT in the pancreas at 1.0hr but not 0.5 and 2.0hr (Tables 1 & 4). Thus, although pancreatic 5HT can be influenced by precursor availability, the factors influencing the levels may be different than those needed to alter brain 5HT. In the pancreas, both the magnitude of change of the precursor and the time course of synthesis, and perhaps release, may be important variables in determining alterations in pancreatic 5HT.

In this paper we have shown that acute intragastric infusion of

protein and carbohydrate can influence both tryptophan and 5HT in the brain and other tissues. The exact effects seen depend on the nature of the transport system for tryptophan and the relationship between tryptophan and 5HT in the different tissues. In the pineal it is known that tryptophan availability can influence 5HT (Young and Anderson 1982) but the tryptophan level seems to be protected from alterations caused by acute dietary intake. Intestinal tryptophan, like the pineal compound, is not subject to influence from the other large neutral amino acids. However, unlike the pineal, it can be influenced by protein intake, because tryptophan in protein is absorbed directly into the intestine. Intestinal 5HT seems to be influenced more by other unknown factors than by precursor availability. Perhaps the most interesting result from this study is that the pancreas, like the brain, is influenced by dietary intake due to competition between the large neutral amino acids for entry into this organ. Pancreatic 5HT can be influenced by changes in tryptophan and thus, is susceptible to dietary influences. The exact role of 5HT in the pancreas is unclear but it does seem to play some role in modulating release of insulin and glucagon (Lebovitz and Feldman 1973, Matsumoto et al. 1984). Whether the changes in pancreatic 5HT play some role in modulating insulin and glucagon profiles after protein or mixed protein and carbohydrate meals remains to be seen.

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TABLE 1. Effect of protein and carbohydrate on 5HT-containing tissues

	TRP (µg/g or ng/pineal)		5HT (µg/g or ng/pineal)	
	control	protein	control	protein
BRAIN	4.35±0.41	2.63±0.69***	0.362 ± 0.031	0.302 ± [illegible]
PANCREAS	5.26±2.21	1.65±0.69***	0.0947± 0.0417	[illegible]
INTESTINE	7.46±2.44	9.68±2.03*	1.87 ± 0.53	[illegible]
PINEAL	8.47±2.05	8.17±2.48	99.5 ±29.2	[illegible]

	TRP (µg/g or ng/pineal)		5HT (µg/g or ng/pineal)	
	control	glucose	control	glucose
BRAIN	3.65±0.47	5.99±0.53***	0.350 ± 0.045	0.425 ± [illegible]
PANCREAS	3.30±0.81	5.02±1.04**	0.0427± 0.0124	0.0447± 0.0071
INTESTINE	8.19±1.69	6.74±0.68*	1.88 ± 0.31	1.98 ± 0.37
PINEAL	7.31±2.48	6.76±2.35	93.6 ±38.9	93.9 ±43.9

Protein (1g) or glucose (2g) were given intragastrically and the rats were killed 2 hr later. Protein and glucose experiments were run on different days. Results in ug/g for brain, pancreas and intestine and in ng/pineal are given as mean of 8 ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, indicate values significantly different from controls.

TABLE 2. Effect of protein on plasma tryptophan

	Portal Blood (µg/ml)		Cardiac Blood (µg/ml)	
	Control	Protein	Control	Protein
0.5hr	10.8±3.4	12.6±1.4	12.1±2.5	12.2±2.3
1hr	13.1±2.3	15.9±3.6	15.4±3.6	16.9±4.2

Protein (1g) was given intragastrically. One half or one hr later the animals were anaesthetized with nembutal (50mg/kg) and blood was taken from the portal vein and heart. Results are given as mean of 8 ± SD. No significant differences were observed.

TABLE 4. Effect of carbohydrate on indoles in the intestine and pancreas

	TRP (µg/g)		5HT (µg/g)	
	control	glucose	control	glucose
INTESTINE				
0.5hr	10.0 ±1.1	10.6 ±2.1	2.71 ±0.36	2.98 [illegible]
1.0hr	9.57±2.47	10.8 ±2.5	2.59 ±0.42	3.[illegible] [illegible]*
PANCREAS				
0.5hr	6.20±1.61	9.92±2.02*	0.0339±0.0158	[illegible]
1.0hr	11.3 ±2.8	15.4 ±3.2*	0.0218±0.0123	[illegible]

The experiment was performed as described in the legend to Table 1 except that the animals were killed at 0.5 or 1.0hr. Results are given as mean of 8±SD *$p<0.05$, **$p<0.01$, indicate values significantly different from controls.

TABLE 5. Effect of valine on 5HT-containing tissues

	TRP (µg/g or ng/pineal)		5HT (µg/g or ng/pineal)	
	control	valine	control	valine
BRAIN	4.22 ±0.84	2.68 ±0.26**	0.270 ±0.038	0.228 ±0.038*
PANCREAS	6.16 ±1.50	4.26 ±1.38*	0.0503±0.0209	0.0537±0.0224
INTESTINE	5.45 ±2.12	5.63 ±2.53	1.55 ±0.37	1.40 ±0.24
PINEAL	12.6 ±7.3	13.3 ±4.5	169±88	111±48

Valine (400 mg/kg) was given intragastrically and the rats were killed 2hr later. Results are given as mean of 8 ± SD. *$p<0.05$, **$p<0.01$ indicate values significantly different from controls.

PREFACE TO CHAPTER THREE

The previous chapter demonstrated that the dietary macronutrients, protein and carbohydrate can influence 5HT metabolism in the pancreas and the brain by altering precursor availability. It was not known whether the increases or decreases in tissue 5HT were a reflection of altered release, that is, whether these changes were of functional significance. As the area of expertise in this laboratory was neurochemistry, and because the role of 5HT in the release of insulin was a matter of some controversy, it was decided to direct our efforts towards determining the functional significance of the changes observed in brain 5HT metabolism.

In this chapter, a new method for the determination of 5HT in rat cerebrospinal fluid was presented and validated. It was hypothesized that 5HT in the CSF would be an index of functional 5HT since its presence in the fluid suggests that it had been released from the neuron and was available for interaction with the post-synaptic receptor. To verify this, drugs known to act on 5HT function were administered and cerebrospinal fluid withdrawn to determine if alterations in CSF 5HT levels reflected the known actions of the agents used.

Chapter 3

SEROTONIN IN CISTERNAL CEREBROSPINAL FLUID OF THE RAT: MEASUREMENT AND USE AS AN INDEX OF FUNCTIONALLY ACTIVE SEROTONIN

ABSTRACT

A simple, selective reverse-phase HPLC-fluorometric method is described for the determination of serotonin (5HT) in cisternal CSF of the rat. The mean (±SE) value of CSF 5HT observed in control adult male rats was 457±83 pg/ml (N = 16). In an attempt to validate the measure as an index of extracellular, or functionally active, 5HT, groups of rats were treated with fenfluramine, amitriptyline, pargyline, pargyline plus tryptophan, and 5-hydroxytryptophan plus carbidopa. In all cases CSF 5HT appeared to reflect well the presumed effects of the agents on extracellular levels of 5HT. CSF 5HT was superior in this regard to brain 5HT, brain 5HIAA, or CSF 5HIAA levels. The measurement of cisternal CSF 5HT would appear to offer a convenient index of functionally active 5HT.

INTRODUCTION

The assessment of central serotonergic function is of critical importance in a wide range of neurochemical studies. Investigations of serotonin's role in the mechanism of drug action, its role in a variety of normal processes, and its possible alteration in neuropsychiatric disorders all require an accurate determination of serotonin (5HT) function. It has been shown that increasing extracellular concentrations of 5HT will increase its effect on neurons possessing 5HT receptors (Bradshaw et al., 1973). Although the specific location, type, and state of the receptors involved will influence the consequences of changes in extracellular fluid (ECF) 5HT concentration, the ECF 5HT concentration is of primary importance. The status of 5HT function and the effect of various agents and actions on 5HT release have been assessed by electrophysiological (Bradshaw et al., 1973; Aghajanian, 1982) behavioral (Green and Grahame-Smith, 1976), neuroendocrine (Fuller and Clemens, 1981), and neurochemical methods. Each of the approaches has its own set of advantages and limitations. The particular pros and cons of the various neurochemical methods employed will be briefly reviewed.

In animal studies investigators have most commonly measured brain levels of 5HT and its major metabolite, 5-hydroxyindoleacetic acid (5HIAA). Complicating the interpretation of these measurements are the facts that most 5HIAA arises from intraneuronal metabolism of unreleased 5HT (Commissiong, 1985; Wolfe et al., 1985), and that nearly all of the 5HT in the brain is found stored within the neuron. In a number of situations measurement of the intraneuronally produced or stored compounds will reflect extracellular pools; however, at times the relationship between brain levels and extracellular levels may be minimal

or even inverted. Alternatively, the turnover of monoamines and/or their metabolites has been determined by blocking their metabolism at some point or by using labeled precursors (Neff, 1972). While these techniques offer a more direct measurement of synthesis rates of the neurotransmitter than steady-state measurements, they remain an indirect and, at times, misleading index of extracellular levels.

Several methods have been used to measure more selectively extracellular species; these include: push-pull cannulae (Myers et al., 1985), in-vivo dialysis (Hutson et al., 1985), in-vivo electrodes (Brazell and Marsden, 1982), and sampling of cerebrospinal fluid (CSF). The push-pull cannula, in-vivo dialysis probe, and in-vivo electrodes can be used to sample ECF in specific brain areas. When the first two of these sampling methods are combined with high-performance liquid chromatography (HPLC), a great deal of selectivity can be obtained. However, with the in-vivo techniques some degree of tissue disruption occurs at the sampling site. This may affect normal neuronal function and, if capillaries are broken, may lead to release of platelet 5HT into the ECF (Echizen and Freed, 1986). Although in-vivo amperometry has provided the best time resolution of all, specificity has remained somewhat problemmatic using in-vivo electrodes (Echizen and Freed, 1982, Mueller et al., 1985).

Finally, rat CSF has been sampled by ventricular cannulae (Le Quan-Bui et al., 1982), cisternal cannula (Hutson et al., 1985), or cisternal puncture (Chaouloff et al., 1986). The disadvantage of the CSF techniques is the more diffuse anatomical origin of the compounds measured. If a more global assessment of ECF concentrations is desirable or sufficient, the CSF techniques offer the advantages of simplicity and

an absence of brain tissue damage. Nearly all the previous studies of animal CSF (and ECF) have concerned the measurement of monoamine metabolites, rather than the monoamines themselves. Serotonin has been measured previously in cat ventricular perfusate (Hammond, 1984) and in ventricular CSF from the rat (Le Quan-Bui, 1982). We present here the first report of 5HT in cisternal CSF of the rat and have attempted to validate the measure as an index of extracellular 5HT.

Methods

Serotonin, 5HIAA, tryptophan, N-methylserotonin (NMS), sodium octyl sulfate, ascorbic acid, pargyline, amitriptyline and DL-5-hydroxytryptophan (5HTP) were purchased from Sigma Chemical Co. (St. Louis, MO). Carbidopa was from Merck Sharp and Dohme and fenfluramine hydrochloride was from A.H. Robins. Glass-distilled acetonitrile was obtained from American Burdick and Jackson (Muskegon, MI). All other chemicals were reagent grade from local suppliers.

Male Sprague-Dawley rats weighing 250-330g were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The animals were maintained on a 12 hr light/12 hr dark cycle for at least 5 days before an experiment. Rats were deprived of food, but not water, beginning at 8:00 am (2 hr. after the onset of the light period) on the day of an experiment. All experimental procedures were carried out between 9:00 am and 12:00 noon. Drugs were dissolved in isotonic saline and injected intraperitoneally in a volume of 5 ml/kg body weight. The following doses were used; pargyline, 70 mg/kg; TRP, 100 mg/kg; fenfluramine, 40 mg/kg; amitriptyline, 50 mg/kg; 5HTP, 200 mg/kg; and carbidopa, 50 mg/kg. Brain and CSF were taken one hour after the drug treatments. The rats were anesthetized with a mixture of ketamine (90

mg/kg) and xylazine (9 mg/kg) and CSF was taken by cisternal puncture as described previously (Young et al., 1980). Brain and CSF samples were stored at -70° until assayed. The drug experiment was performed over two days with a separate control group on each day. CSF specimens were prepared for analysis by adding 100 µl of freshly thawed CSF to a polypropylene tube containing 0.5 ng of NMS (5 µl of 0.1 ng/l) and 10 µl of 25% ascorbic acid.

Brain samples were prepared, after weighing, by adding brains to a plastic tube along with 4.5 ml of 0.1 M $HClO_4$ containing 2000 ng NMS (20 µl of 100 ng/µl NMS), 0.05% metabisulfite, 0.25% ascorbic acid, and 0.1% disodium EDTA. Samples were homogenized on ice using a Bronson Polytron Sonicator (microtip, setting 5, 2 x 15 sec). One half ml of 3.4M $HClO_4$ was added, after brief vortex mixing and sitting on ice for 10 min., tubes were centrifuged (5000 x g) and a portion of the clear supernate removed and stored at -70°C.

Cerebrospinal fluid (25-50 µl) and brain samples (25 µl) were analyzed by injection on an HPLC fluorometric/electrochemical system. The HPLC-F/EC system used for the majority of the work consisted of a Kratos Spectroflow 400 pump (Kratos Analytical Instruments, Ramsey, NJ), Rheodyne 71-25 injector, 5µ Altex Ultrasphere C_{18} column (0.46x25 cm) (Ralnen Instruments, Woburn, MA) a Shimadzu RF-350 fluorometric detector (Shimadzu Instruments, Columbia, MD) and a Bioanalytical Systems (West Lafayette, IN) electrochemical detection system (TL-4 glassy carbon electrode, Ag/AgCl reference electrode. LC-2A controller). Excitation and emission wavelengths (20 nm bandpass) were set at 285 and 340 nm, respectively; the working electrode potential was set at +0.70V. A mobile phase of 88% pH 4.4 0.02M sodium acetate containing 70 mg/l sodium

octyl sulfate, 240µl/l of triethylamine, and 25 mg/l of Na_2EDTA; 12% acetonitrile was used to determine 5HT, 5HIAA, TRP, and NMS in both CSF and brain specimens (1.5 ml/min).

RESULTS

As shown in Figure 1, 5HT, 5HIAA, tryptophan (TRP), and the internal standard, N-methylserotonin (NMS) were determined in rat cisternal CSF by directly injecting 25-50 µl of CSF on a HPLC-fluorometric system. Serotonin was determined fluorometrically with an absolute detection limit of 4 pg, equivalent to a concentration detection unit (S/N=2) of 80 pg/ml when 50 ul of CSF was injected. Samples (N=3-4) were determined (HPLC-F) with within-day coefficients of variation of 7.0% (5HT), 2.7% (5HIAA), and 1.7% (TRP). Day-to-day coefficients of variation of 4.4%, 2.9%, and 6.7% were observed for 5HT, 5HIAA, and TRP, respectively. Added 5HT, 5HIAA, TRP, and NMS were recovered quantitatively (>95%). The identities of the 5HT, 5HIAA, and TRP peaks were confirmed by reinjecting CSF samples on the LC-F/EC system while eluting with mobile phases having increased octyl sulfate concentrations and/or decreased pH. In all cases sample peaks coeluted with the appropriate standard peak. The hydroxyindoles could also be determined by HPLC-amperometry and the excellent agreement observed between fluorometric and amperometric values for 5HT ($r = 0.996$, $p<0.001$) and for 5HIAA ($r = 0.998$, $p<0.001$) further confirms their identities as do the changes seen after pharmacological treatments. Similar results were obtained when the method was used to determine 5HT, 5HIAA, and TRP in brain, although average recovery of the internal standard (NMS) was $79.2 \pm 5.7\%$ (N=17).

Group mean values observed for CSF 5HT, 5HIAA, and TRP in control and treated animals are presented in Table 1. For purposes of analysis, the

control groups sacrificed on Day 1 and Day 2 are separated; however, no significant differences were seen between the two control groups. A mean (± SE) 5HT concentration of 457±83 pg/ml was observed in cisternal CSF obtained via cisternal puncture from untreated adult (control) Sprague-Dawley rats (N = 16).

Mean values for the other compounds (5HIAA, TRP) in CSF and for the three indoles in brain (see Table 1 and 2) were in good agreement with previous reports. Somewhat surprisingly, intra-compound correlations between CSF and brain concentrations in control animals all were nonsignificant. Significant correlations were observed between brain 5HT and CSF 5HIAA ($r=0.75$, $p<0.001$), between CSF TRP and CSF 5HT ($r=0.6$, $p=0.05$), and between brain TRP and brain 5HIAA ($r = 0.70$, $p<0.001$).

Group mean concentrations observed for 5HT, 5HIAA, and TRP in CSF and brain of animals treated with a range of agents are presented in Tables 1 and 2. In pargyline-treated rats CSF 5HT increased 277% ($p = 0.02$), while CSF 5HIAA declined (-22%) and TRP increased (30%) only slightly (and non-significantly). Increases in brain levels of 5HT (203%, $p<0.01$) were similar to those seen in CSF; however, the decline seen for brain 5HIAA was more pronounced (-55%, $p<0.01$) than observed in CSF. When TRP was administered with pargyline, a much larger increase in CSF 5HT was observed (21-fold vs. 2.8-fold) compared to pargyline alone. The increase in brain 5HT after TRP + pargyline was only slightly greater (246% vs. 203%) than with pargyline alone. Smaller declines were seen for 5HIAA in both CSF and brain, although the decline seen in brain was significant (-51%, $p<0.01$). In both CSF and brain, TRP was much higher--10.8fold and 9.3-fold ($p<0.001$), respectively--in animals receiving pargyline + TRP versus pargyline alone.

Animals treated with fenfluramine exhibited a large increase in CSF 5HT (16.3-fold, $p < 0.001$), while brain levels declined (-55%, $p < 0.01$). CSF levels of 5HIAA increased significantly (73%, $p < 0.01$), although brain levels of 5HIAA were unchanged. Brain and CSF TRP also were both significantly increased after fenfluramine (+23%, $p < 0.01$; +72%, $p < 0.001$).

Treatment with amitriptyline caused a 333% ($p < 0.01$) increase in CSF 5HT without affecting brain 5HT levels. Levels of 5HIAA and TRP in CSF and brain were unchanged, compared to controls after amitriptyline. Finally, administration of 5-hydroxytryptophan (5HTP) and carbidopa resulted in a large increase in CSF 5HT (17.5-fold, $p < 0.001$), as well as an increase in brain 5HT (+331%, $p < 0.05$). Treatment with 5HTP also caused marked elevations of CSF 5HIAA (11-fold, $p < 0.001$) and brain 5HIAA (7.9-fold, $p < 0.001$). In addition, a small, but significant, increase also was seen for brain TRP (+28%, $P < 0.001$).

DISCUSSION

This first report of 5HT levels in cisternal CSF of the rat appears to be in approximate agreement with previous reports of 5HT levels in human and animal CSF. Values in the monkey have been reported to range from 20-640 pg/ml (Taylor et al., 1985), a mean of 4.8 ± 27 ng/ml has been reported from canine lumbar CSF (Bardon and Ruckrebusch, 1984), and a mean of 5.1 ± 1.2 ng/ml was observed in rat ventricular CSF (Le Quan-Bui et al., 1982). Reported mean levels of 5HT in human lumbar CSF vary widely. Although several recent reports of approximately 1 ng/ml (Linnoila et al., 1986; Koerber et al., 1984; Narasimhachari, 1984; Koskiniemi, 1985) are lower than most previous determinations, the observations of Tyce et al, (80 ± 40 pg/m) (1985), Anderson (81 ± 13 pg/ml) unpublished data) and

Mefford (<100 pg/ml) (personal communication) indicate that the true value might be even lower. Some of the variation seen, at least in human CSF, probably can be attributed to a lack of analytical specificity. As mentioned, mean values for the other compounds (5HIAA, TRP) in CSF and for all three indoles in brain (Table 2) were in good agreement with previous reports of their levels in the rat (Young et al., 1980; Koerber et al., 1984; Anderson and Purdy, 1969; Boulton et al., 1985).

Marked changes in CSF 5HT levels were seen after administration of pargyline, (a monoamine oxidase inhibitor (MAOI), pargyline plus TRP (MAOI plus precursor), fenfluramine, (primarily a 5HT releaser, with 5HT reuptake inhibiting and tryptophan hydroxylase inhibiting properties (Knapp and Mandell, 1976), amitriptyline (a reuptake inhibitor), and 5HTP (a precursor). The increases were especially marked (16 to 21-fold) after fenfluramine, pargyline plus tryptophan, and 5HTP administration. The greater potency of these agents in increasing CSF 5HT is in concordance with their relative potency as determined by behavioral (Green and Grahame-Smith, 1976) and neuroendocrine studies (Fuller and Clemens, 1981). In particular, the relative increases seen in CSF 5HT after pargyline alone (2.8-fold) and after pargyline + TRP (21-fold) are consistent with studies showing that an MAOI + TRP, unlike an MAOI alone, produces pronounced behavioral activation in the rat (Green and Grahame-Smith, 1976). Parenthetically, the large difference in CSF 5HT levels, and the similarity in brain 5HT levels, seen after these two treatments tends to support the idea (Green and Grahame-Smith, 1976) of 5HT spillover. It appears that much of the increase in brain 5HT seen after inhibition of MAO can be stored intraneuronally. However, when TRP is also administered, further increases resulting from increased synthesis

of 5HT can no longer be accommodated.

Treatment with fenfluramine or 5HT + carbidopa, which produced increases in CSF 5HT similar to that seen with an MAOI + TRP also causes marked behavioral activation (the serotonin syndrome) in the rat (Modigh, 1976; Trulson and Jacobs, 1976). While amitriptyline and pargyline (neither of which produce the behavioral syndrome) caused increases in CSF 5HT of 2.8 and 3.3-fold, respectively, the elevations are clearly much less than those seen with the behaviorally-activating agents.

When one examines the changes in CSF 5HT, CSF 5HIAA, brain 5HT, and brain 5HIAA after drug treatment it is apparent that, in nearly every case, CSF 5HT is superior to the other measures in reflecting changes in functionally active 5HT. After treatment with fenfluramine CSF 5HIAA increased slightly while brain 5HIAA was unchanged, and brain 5HT actually declined by over 50%. However, the large increases in CSF 5HT clearly reflected the potent serotonergic activation caused by fenfluramine. When amitriptyline was administered, CSF and brain 5HIAA, as well as brain 5HT, were unchanged. An increase in CSF 5HT (2.8-fold) was the only indication that amitriptyline acts to increase serotonergic function. As expected, brain 5HIAA decreased (decreases in CSF 5HIAA were not significant) and brain 5HT increased after pargyline or pargyline plus TRP. The effects on 5HIAA are directly opposite to the treatments' effect on serotonin function and, as mentioned, the changes in brain 5HT are not proportional to the changes observed in functional activity.

It would appear, from the data presented, that CSF 5HT is superior to CSF 5HIAA, brain 5HIAA, or brain 5HT in assessing changes in functionally active 5HT. This is because the latter measures do not

always reflect changes in the compartmentalization of 5HT. It remains to be seen whether the changes that occur in CSF 5HT are of similar absolute magnitude to those that occur in ECF 5HT. This question should be resolved when problems with in-vivo push-pull cannulization, in-vivo dialysis and in-vivo amperometry are resolved. In the long run, a variety of approaches taken in conjunction with each other will supply the most information. Meanwhile, there is good circumstantial evidence that CSF 5HT reflects the brain ECF level.

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Table 1

Cisternal CSF concentrations[+] of 5HT, 5HIAA, and TRP in control and treated rats

Treatment	N	5HT	5HIAA	TRP
Control (day 1)	6	0.505±0.327	59.6± 16.9	253± 54
Pargyline	5	1.40 ±0.68*	56.4± 15.3	329± 120
Pargyline + TRP	8	10.5 ±6.2***	50.5± 14.2	3540±1600***
Fenfluramine	10	8.25 ±5.04***	103 ± 29**	434± 85***
Control (day 2)	10	0.436±0.351	68.9± 18.1	329± 75
Amitriptyline	8	1.45 ±0.78**	79.9± 21.2	331± 80
5-HTP + carbidopa	6	7.61 ±4.32***	754 ±245***	403± 91
Combined Control Group	16	0.457± .331	65.4±17.4	301± 76

+ mean ± SD, CSF units ng/ml, brain units ng/g.
*p<.05, **p<.01, ***p<.001 for Student's t-test versus within-day control group.
See Methods section for details of experimental design and methodology.

Table 2

Brain concentrations+ of 5HT, 5HIAA, and TRP
in control and treated rats

Treatment	N	5HT	5HIAA	TRP
Control (day 1)	12	561± 67	292± 38	4750± 582
Pargyline	11	1140±776*	130± 43*	[illegible]
Pargyline + TRP	11	1380±253***	142± 15*	[illegible]
Fenfluramine	11	252± 64**	320± 81	[illegible]
Control (day 2)	11	556± 59	310± 30	[illegible]
Amitriptyline	12	579± 63	272± 30	[illegible]
5-HTP + carbidopa	12	1840±386**	2460±756***	6780± [illegible]
Combined Control Group	23	559±62	301±35	5020± 63

+ mean ± SD, CSF units ng/ml, brain units ng/g.
*p<.05, **p<.01, ***p<.001 for Student's t-test versus within-day control group.
See Methods section for details of experimental design and methodology.

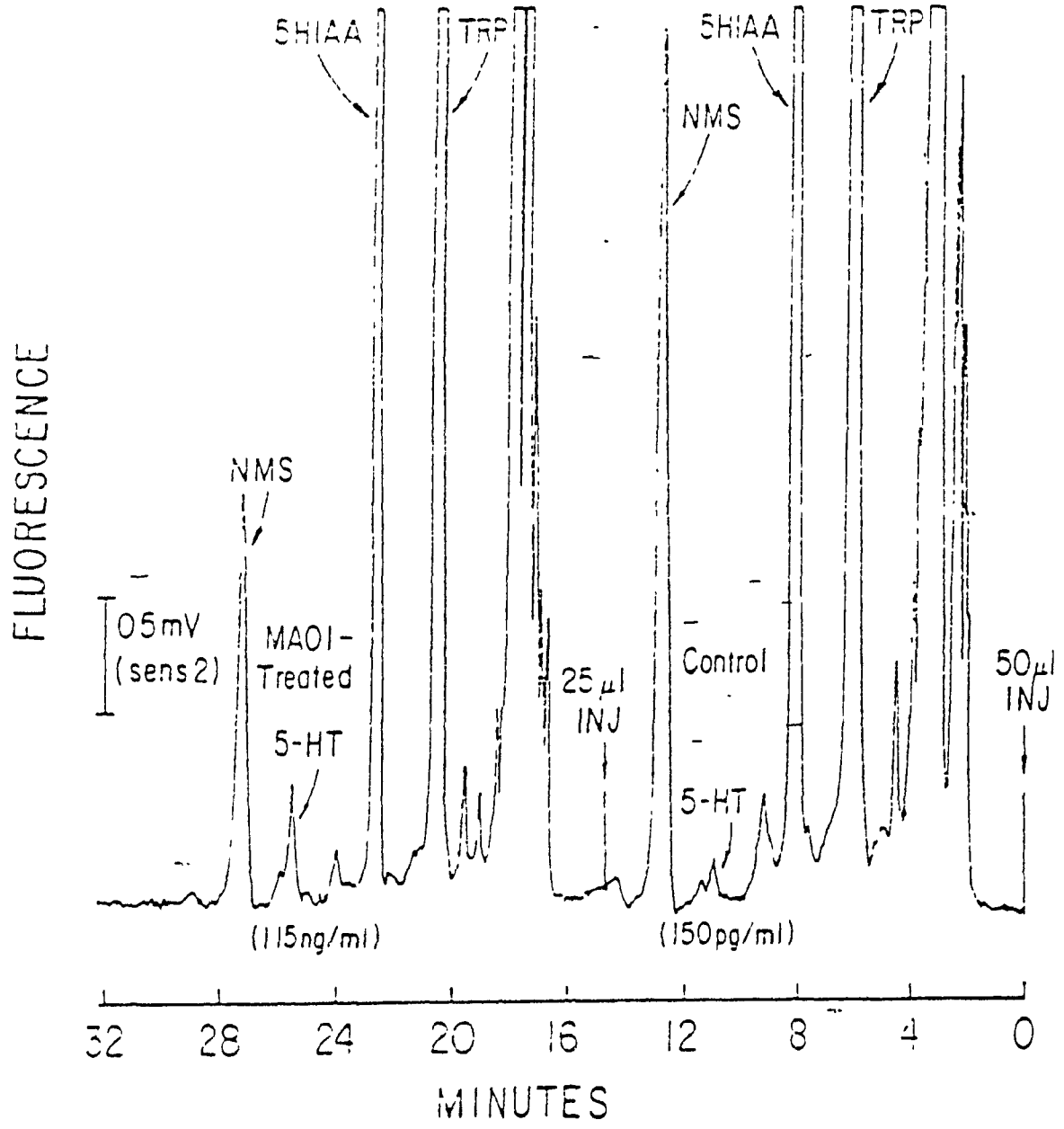

Fig. 1 The HPLC-fluorometric determination of 5HT, 5HIAA, TRP, and NMS (internal standard, 5 ng/ml) in cisternal CSF of control and MAOI-treated rats. The level of 5HT in the control CSF sample shown is in the low end of the normal range and is about twice the detection limit of 80 pg/ml.

PREFACE TO CHAPTER FOUR

Chapter two demonstrated that brain 5HT metabolism was altered by dietary intake. It was questioned whether these changes were correlated to the amount of 5HT released or whether they were simply alterations in 5HT synthesis regulated by precursor availability. Having validated a methodology for the measurement of rat CSF 5HT which was used as an index of functionally active 5HT (chapter three), we were then in a position to assess the functional significance of the changes observed in brain 5HT.

Evidence in the literature suggested that, in some circumstances, even tryptophan loading did not increase measurements of functional 5HT. If a doubling of brain 5HT, as would occur after tryptophan administration, could not increase CSF 5HT, then it would be unlikely that dietary intake, a relatively subtle manipulation, would be effective. Therefore, it was necesary to first determine the effect of tryptophan administration on CSF 5HT. This was done under normal lighting conditions and also using a behavioral arousal paradigm that involved placing the animals in the dark in a new environment for a brief period. Previous studies had suggested that increasing arousal can increase the firing rates of serotonergic neurons, thereby enhancing the likelihood of increases in CSF 5HT. Once this was established, the effect of protein and carbohydrate on CSF 5HT was also examined, using repeated administrations of the macronutrients to optimize effects. Thus, an attempt was made to ascertain whether the changes observed in brain 5HT after dietary treatments were of functional importance, by the measurement of CSF 5HT.

Chapter 4

THE EFFECT OF TRYPTOPHAN, PROTEIN AND CARBOHYDRATE ON 5-HYDROXYTRYPTAMI SYNTHESIS AND RELEASE IN THE RAT

ABSTRACT

Acute administration of tryptophan failed to increase cerebrospinal fluid (CSF) 5-hydroxytryptamine (5HT), an index of 5HT release, when rats were treated in their home cage in the light. The experiment was repeated, but the animals were placed in new cages in a dark room, a treatment designed to increase their arousal and therefore increase the firing rate of 5HT neurons. Under these circumstances tryptophan increased CSF 5HT. When rats were given repeated doses of protein or carbohydrate and aroused there was no increase in CSF 5HT. Our results suggest that diet mediated changes in brain 5HT are too small to cause appreciable alterations in 5HT function, but that tryptophan can increase 5HT release under suitable circumstances.

INTRODUCTION

The role of substrate availability in the regulation of serotonin (5HT) synthesis has received much attention over the last twenty years. Increasing plasma tryptophan, will increase brain levels of 5HT as well as the concentration of its major metabolite, 5-hydroxyindoleacetic acid (5HIAA) (Ashcroft et al., 1965). Dietary treatments have also been used to alter tryptophan availability. As tryptophan competes with other large neutral amino acids for transport across the blood-brain barriers (Oldendorf and Szabo, 1976), increasing or decreasing the plasma concentration of the competitors influences brain tryptophan and 5HT (Fernstrom et al, 1975). Thus, carbohydrate, which lowers the concentration of the competitors in plasma, due to the insulin-mediated uptake of the branched-chain amino acids into muscle, can increase brain tryptophan and 5HT (Fernstrom and Wurtman, 1971), while protein has been shown to decrease brain tryptophan and 5HT (Teff and Young, 1988).

Although these results indicate that 5HT synthesis and levels are affected by tryptophan availability, it does not necessarily imply that the amount of 5HT released into the extracellular fluid (ECF) has been altered. The concentration of 5HT in the ECF, which is available for interaction with the post-synaptic receptor, must be considered as functionally active 5HT. Attempts have been made to quantitate ECF 5HT after tryptophan administration, using a variety of methods, each of which has it's inherent advantages and disadvantages. Results from these studies are contradictory. In some cases, the amount of 5HT released or indices of functional 5HT were not altered by tryptophan administration (De Simoni et al., 1987; Marsden et al., 1979), while, in others, increases were demonstrated (Ternaux et al., 1976; Hutson et al., 1985).

In the experiments presented here, we have hypothesized that the effect of altered precursor availability on 5HT release will be greater when the neuron is actively firing. This concept is based on evidence demonstrating that the firing rate of dorsal raphe neurons is a function of behavioral state (Trulson and Jacobs, 1979). Thus, neuronal activity is high during active waking and declines as the animals progress through the various stages of drowsiness to sleep.

We have recently reported a method for the direct determination of 5HT in the cisternal cerebrospinal fluid of the rat and validated the use of this measurement as an index of functionally active 5HT (Anderson et al., 1987). In this paper, we have examined the effect of tryptophan administration on CSF 5HT at two different levels of arousal of the animals, to determine if precursor availability can increase functionally active 5HT. We have also looked at the effect of the dietary macronutrients protein and carbohydrate, to see whether the changes in the brain observed after administration of these compounds is accompanied by changes in functionally active 5HT.

METHOD

Tryptophan, 5HT and 5HIAA were purchased from Sigma Chemical Co. (St. Louis, MO).

Male Sprague-Dawley rats weighing 150-170g were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The animals were maintained on a 12 hr light dark cycle with lights on at 6:00 am for at least one week before the experiment, during which time they were handled daily to minimize stress effects. The night before the experiment (17:00) rats were deprived of food, but not water until 9:00 am the following morning. All experiments were performed between 9:00 am

and 12:00 noon. In the first experiment, rats were administered either tryptophan (400 mg/kg) or water intragastrically in a total volume of 4 ml. Half of the animals were then placed into a clean cage with fresh bedding material and brought into a dark room. Both the change in environment and the darkness were designed to be mildly arousing. The remaining animals were returned to their home cages. Two hours after treatment, animals were anaesthetized with a mixture of ketamine (90 mg/kg) and zylazine (9 mg/kg) (note: control animals in the dark were more sensitive to the anaesthetic and approximately half the concentration was required in these animals) and CSF was taken by cisternal puncture as previously described (Young et al., 1980). In the second experiment, multiple doses of the dietary macronutrients were used in order to maximize effects. Therefore, 2 ml of protein (50% weight/volume of egg albumin) or 2 ml of carbohydrate (50% weight/volume of glucose) were administered intragastrically, at 9:00, 9:30 and 10:00 am. After the first intubation, animals were placed in the dark room. Two and one half hours after the initial treatment, animals were anaesthetized as described above. After withdrawal of CSF, brains were removed and both samples placed on ice and frozen.

Brain and CSF samples were stored at -70°C until assayed. CSF specimens were prepared for analysis by adding 100 ul of freshly thawed CSF to a polypropylene tube containing 10 µl of 25% ascorbic acid. Brains were weighed, then homogenized on ice in a 25% weight/volume solution of 0.4M perchloric acid with 0.25% ascorbic acid using a Polytron homogenizer. Samples were then centrifuged for 15 minutes at 6,000 x g. After centrifugation, an aliquot for the supernatant was injected directly into a high performance liquid chromatograph. Brain tryptophan,

5HT and 5HIAA were determined by the method of Anderson et al. (1981). Briefly, this employs a Waters C18-reverse phase column with fluorimetric detection. The mobile phase consisted of 88% 0.01M sodium acetate buffer at pH 4.25 and 12% methanol. CSF tryptophan, 5HT and 5HIAA were measured by the method of Anderson et al. (1987). A 5u Altex Ultrasphere C18 column (0.46 x 25 cm) was used. In this experiment, the mobile phase was 88% of pH 4.4.02M sodium acetate containing 140 mg/l sodium octane sulfonate, 240 ul/l of triethylamine and 25 mg/l of EDTA. Added to this was 12% acetonitrile. A Shimadzu RF-530 fluorometer was used in both assays.

In the first experiment, statistical analysis was performed using a two-way ANOVA, with differences between group means being determined by Tukey's test. In the dietary experiment, differences between the two treatments was performed using a Student's t test. In both cases, $p<0.05$ was considered to be significant.

RESULTS

Table 1 illustrates the effect of tryptophan administration on CSF tryptophan, 5HT and 5HIAA in control and treated animals, two hours after treatment. As previously reported (Young et al., 1980), tryptophan administration increased tryptophan and 5HIAA in cisternal CSF of the rat. This effect was independent of the lighting conditions. CSF tryptophan was increased 21-fold and 25-fold in the light and the dark ($p<0.001$ for both treatments) respectively. 5HIAA in the CSF was elevated approximately 2-fold ($p<0.001$). In contrast, the effect of intragastric tryptophan on CSF 5HT was dependent on the environmental conditions in which the rat was placed. When the rat was maintained in the home cage under normal lights, tryptophan administration had no

effect on CSF 5HT. If the rats were placed in a dark room immediately after treatment, a significant increase in CSF 5HT was observed ($p<0.01$). The ANOVA revealed a significant environment x treatment interaction ($F = 8.58$, df 1,23; $p<0.01$). There was no difference between the means of the two control groups (i.e. water:light vs. water:dark).

A significant elevation in brain tryptophan, 5HT and 5HIAA was seen in the carbohydrate treated animals relative to protein administration (Table 2). A 52% increase ($p<0.001$) in brain tryptophan resulted in a rise of 10% and 17% of 5HT ($p<0.02$) and 5HIAA ($p<0.01$), respectively in the brain. Carbohydrate significantly increased CSF tryptophan by 40% ($p<0.001$) relative to protein. Though a trend to an increase in the mean was exhibited in CSF 5HT after carbohydrate, no significant difference was found. Similarly, CSF 5HIAA was not significantly altered.

DISCUSSION

The present experiments demonstrate that under specific environmental conditions which increase arousal, tryptophan administration can alter functionally active 5HT, as reflected by increases in CSF 5HT levels. The dietary macronutrients, protein and carbohydrate had no effect on CSF 5HT despite significant changes in brain 5HT. This suggests that extreme increases in precursor availability can, in some circumstances, increase functional 5HT, but that the effects of dietary intake on central 5HT are metabolic and not functional.

It has been consistently demonstrated that tryptophan administration increases 5HT synthesis and metabolism. Intraperitoneal injection or dietary administration of tryptophan increases brain 5HT and 5HIAA (Eccleston et al., 1965). CSF 5HIAA is also elevated by both treatments

(Modigh, 1975; Young et al., 1980). Though changes in 5HIAA are usually interpreted as being an indication of 5HT metabolism, they are not necessarily representative of 5HT release or function. Behavioral testing, correlated with biochemical data, suggests that to some extent, 5HIAA is derived from the intraneuronal metabolism of 5HT which has never been released (Green and Grahame-Smith, 1976; Wolf et al., 1985). Therefore, one cannot conclude that the changes observed in brain and CSF 5HIAA after tryptophan administration parallel changes in functional 5HT.

Direct measurement of released 5HT has been hampered by methodological problems. In many cases, techniques lack the specificity to differentiate between 5HT and 5HIAA or do not possess the sensitivity to detect the low levels of 5HT present in the extracellular fluid or CSF. In vivo voltammetry has been used to monitor the effects of tryptophan administration on 5HT release, though the oxidation peak being measured is most likely derived frm both 5HIAA and 5HT (Echizen and Freed, 1986). In two separate experiments tryptophan did not increase current values in the striatum of the rat (Marsden et al., 1979; De Simoni, 1987). Other methods have shown more positive results. Perfusion of the lateral ventricle in the rat, resulted in a significant, but short lasting increase in the amount of 5HT released during the second hour after acute tryptophan treatment. In the experiments presented here, the direct measurement of 5HT in the CSF has been used as an index of functionally active 5HT. The validity of this technique has been verified in earlier work (Anderson et al., 1987). In the present study we found that when tryptophan was given to rats in the light in their home cage (i.e. at low arousal) there was no effect on CSF 5HT despite large increases in CSF tryptophan and a doubling of 5HIAA.

Therefore, under these circumstances, precursor availability is not a regulating factor in 5HT release. However, if the animals were placed in a new cage in a darkened room during their normal "lights on" period, a procedure designed to increase their level of arousal, an increase in CSF 5HT was observed.

Behavioral arousal has been correlated with dorsal raphe neuronal activity in cats (Trulson and Jacobs, 1979). High discharge rates were found during active waking or immediately following an auditory stimulus. Low discharge rates were exhibited during drowsiness and various stages of sleep. Release of endogenously formed 5HT is dependent on neuronal activity (Hery et al., 1979). Obviously when 5HT neurons are not firing there is no scope for tryptophan to increase 5HT release. At high levels of arousal when 5HT neurons are firing at a high rate, tryptophan will only increase 5HT release if increased 5HT synthesis enlarges the size of that fraction of 5HT which is releasable. Our results suggest that this is in fact the situation.

A substantial amount of research has been devoted to defining the effects of diet on 5HT metabolism. As tryptophan is an essential amino acid, brain 5HT levels are dependent on dietary intake and tryptophan availability. Generally, protein and carbohydrate exert opposite effects on brain 5HT. In some circumstances, protein lowers brain tryptophan (Glaeser et al., 1983) and 5HT (Teff and Young, 1988) while carbohydrate can increase the two compounds (Fernstrom and Wurtman, 1971). Monitoring of activity levels (Chiel and Wurtman, 1981) or food selection (Li and Anderson, 1982) in rats after dietary manipulation, has suggested that acute food intake can alter behavior. In many cases, it has been implied that the behavioral changes observed are mediated by 5HT. If this were

the case, then a change in functional 5HT would have to occur. Trulson (1985) compared the effects of three different diets on the electrophysiological activity of serotonergic neurons and on the amount of labelled 5HT released after the injection of labelled tryptophan. Neither approach resulted in any significant differences between treatments, suggesting that dietary manipulation did not alter functional 5HT. Our results support this hypothesis. We tested the effects of diet under those conditions which we considered were most likely to produce a change in 5HT function. Thus, we tested the animals after several doses of either protein or carbohydrate, to ensure that brain 5HT was different in the two groups (Table 2), and tested the animals after placing them in the dark in a novel environment to arouse. Although increased arousal resulted in elevated CSF 5HT after tryptophan administration, the relatively modest changes in brain tryptophan and 5HT that occurred after feeding was not accompanied by any arousal-induced elevation of CSF 5HT.

The results of the dietary experiment suggest that protein or carbohydrate meals do not result in any large or generalized alterations in 5HT release. One of the disadvantages in using CSF 5HT as an index of functional 5HT is its lack of neuroanatomical specificity. It may be that 5HT release is altered by food in one or more specific brain areas, but such changes, if they occur, are not as extensive as changes in brain 5HT levels. The simple idea that diet-induced changes in brain tryptophan levels will lead to a generalized alteration in 5HT function is not tenable. Even if alterations in tryptophan availability are related to alterations in 5HT function in specific brain areas, unknown factors must account for regional specificity. Thus, our results suggest that 5HT is not the dominant factor in food-mediated changes in behavior.

Whether 5HT is involved at all and what other systems are involved remains to be seen.

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Table 1. Effect of tryptophan on rat cisternal CSF indoles

Treatment		N	Tryptophan µg/ml	5HT ng/ml	5HIAA ng/ml
Light	saline	8	0.542±0.161	2.31±1.09	144±25
Light	tryptophan	8	12.7 ±2.1	2.13±0.71	393±39
			<0.001	NS	<0.01
Dark	saline	4	0.542±0.090	1.18±0.32	143±20
Dark	tryptophan	7	14.2 ±2.1	2.87±0.63	468±46
			<0.001	<0.01	<0.01

Values are given as mean ± SD. P values relative to relevant control (saline-treated) values are shown. Experimental details are given in the methods section.

Table 2. Effect of intragastric protein or carbohydrate on brain and CSF indoles.

		Brain		
Treatment	N	Tryptophan µg/g	5HT ng/g	5HIAA ng/g
Protein	8	3.80±0.39	397±25	224±23
Carbohydrate	11	5.80±0.98	437±36	263±31
		<0.001	<0.05	<0.01
		CSF		
		Tryptophan ng/ml	5HT ng/ml	5HIAA ng/ml
Protein	8	453±50	1.39±0.35	138±19
Carbohydrate	11	635±113	1.67±0.61	153±27
		<0.001	NS	NS

Values are given as mean ± SD. P values of difference between protein and carbohydrate treated groups are shown. Experimental details are given in the methods section.

PREFACE TO CHAPTER FIVE

In the following three chapters, which involve human subjects, two general questions are being asked; 1) Can the macronutrients, protein and carbohydrate alter 5HT metabolism and function in humans? and 2) Is the role of 5HT in the regulation of food intake of physiological importance in humans? The first question follows the line of the previous animal experimentation, where it was demonstrated that though protein and carbohydrate can effect brain 5HT metabolism (chapter two), these dietary constituents did not alter functional 5HT as measured by PRL or [illegible] four), an index of functional 5HT (chapter three).

The first of the human experiments in this chapter examines the effect of lowering plasma tryptophan on food selection in normal [illegible] males. The administration of an amino acid mixture deficient in tryptophan brings about a substantial decline in plasma tryptophan of a magnitude not observed after dietary intake. Due to the specificity of the treatment, a change in behavior, monitored by a change in food selection would suggest that an alteration in functional 5HT took place, due to diminished availability of tryptophan. In addition, it would confirm the role of 5HT in the regulation of macronutrient selection in humans.

Chapter 5

THE EFFECT OF LOWERING PLASMA TRYPTOPHAN ON FOOD SELECTION IN NORMAL MALES

ABSTRACT

The effects of a tryptophan deficient amino acid mixture on food selection was studied using a double-blind counterbalanced crossover design in normal male subjects. The subjects ingested tryptophan deficient or nutritionally balanced amino acid mixtures in the morning after an overnight fast. Five hours after the tryptophan deficient amino acid mixture plasma tryptophan was only 19% of the level found after the nutritionally balanced amino acid mixture. After [illegible] subjects were allowed to select lunch from a buffet. The [illegible] deficient mixture was associated with a modest but significant [illegible] protein selection with no significant alteration in selection of carbohydrate, fat or total kcal. Our results suggest that 5-hydroxytryptamine is involved in the control of protein selection in humans.

INTRODUCTION

There is a large literature concerned with the effect of altered 5-hydroxytryptamine (5HT) function on feeding in experimental animals (Anderson, 1979; Blundell, 1984; Wurtman and Wurtman, 1984). Results vary somewhat depending on the particular experimental paradigm, but in general 5HT acts to suppress food intake. Fluctuations in 5HT can also, in some circumstances, be involved in the modulation of macronutrient selection, with elevated 5HT increasing protein intake or decreasing the ratio of carbohydrate to protein in the diet.

The possible involvement of 5HT in macronutrient selection may help to explain why 5HT is altered by dietary intake (Anderson, 1979; Blundell, 1984; Wurtman and Wurtman, 1984). The rate of synthesis of 5HT depends in part on the level in brain of its precursor tryptophan. The effect of acute dietary intake on brain tryptophan is not what might intuitively be expected. Thus, ingestion of protein, which contains tryptophan, lowers rat brain tryptophan and 5HT. This is because all the large neutral amino acids compete for transport across the blood brain barrier. Protein ingestion increases plasma levels of the other large neutral amino acids more than tryptophan. This increased competition for the transport system results in a lowering of tryptophan in the brain. On the other hand carbohydrate, which contains no tryptophan causes an increase in brain tryptophan and 5HT. This is because insulin enhances uptake of branched chain amino acids into muscle, thus decreasing their plasma level and competition at the blood brain barrier. As protein and carbohydrate have opposite effects on brain 5HT, and 5HT may control relative intakes of protein and carbohydrate, 5HT may be part of a system ensuring that an animal takes in adequate supplies of both these

macronutrients.

A possible role of 5HT in food selection in humans has been studied by giving tryptophan. Tryptophan was given over a 2-week period to obese subjects with a craving for carbohydrate snacks. It significantly diminished carbohydrate intake in three of the eight subjects, and increased it in one subject. It did not significantly modify snacking patterns in the group as a whole (Wurtman et al., 1981). Tryptophan was also tested against placebo in a double-blind crossover study of eight refractory carbohydrate-craving obese subjects in a weight loss program. Weight loss during the six weeks on tryptophan was not significantly different from the six weeks on placebo (Strain et al., 1985). Tryptophan was also tested against placebo in a double-blind crossover study in healthy lean men. Tryptophan in doses from 1 to 3g, or placebo, was given 45 mins. before subjects selected from a buffet luncheon. Whereas 1g had no effect, both 2 and 3g reduced total calorie intake significantly by 13 to 20% (Hrboticky et al., 1985). In a double-blind comparison of the effects of tryptophan (0.5g) or placebo on various measures in a questionnaire, tryptophan had no effect on the subjects' own rating of their hunger or carbohydrate/protein preference, even though they found tryptophan more sedating (Leathwood and Pollet, 1983). Only one study found an effect of tryptophan on selection of macronutrients in humans (Blundell and Hill, in press). Tryptophan at a dose of 1g or placebo was given with a high protein or high carbohydrate lunchtime meal. Food intake was measured at a free selection test meal three hours later. Tryptophan did not influence total food intake, but caused a significant decrease in carbohydrate selection. This effect was seen when the tryptophan was given with the high protein meal, but not

when it was given with the high carbohydrate meal.

Recently we have developed a method for lowering tryptophan levels acutely in humans by giving a tryptophan deficient amino acid mixture orally. This causes a rapid lowering of mood (Young et al., 1985) an effect consistent with a decrease in 5HT function. We have now looked at the effect of lowering tryptophan on food selection in normal male subjects.

METHOD

The subjects were 22 normal males between the ages of 18 and 30 years, who were recruited through newspaper advertisements. All had at least a high school education, no history of psychiatric disorder or food allergies and were within 10% of their ideal body weight for their height. They were not taking any prescription medication and found the foods used in the study acceptable.

A double-blind counterbalanced cross-over design was used. Each subject came into the laboratory on two days, one week apart. On each occasion they ingested an amino acid mixture orally five hours before selecting lunch from a buffet. Half the subjects received a tryptophan-deficient (T-) amino acid mixture on the first visit and a nutritionally balanced (B) mixture on the second visit. The other half received the mixtures in reverse order. The tryptophan-deficient mixture consisted of L-alanine, 2.75g; L-arginine, 2.45g; L-cysteine, 1.35g; glycine, 1.6g; L-histidine, 1.6g; L-isoleucine, 4.0g; L-leucine, 6.75g; L-lysine monohydrochloride, 5.5g; L-methionine, 1.5g; L-phenylalanine, 2.85g; L-proline, 6.1g; L-serine, 3.45g; L-threonine, 3.25g; L-tyrosine, 3.45g; and L-valine, 4.45g, for a total of 50g of amino acids. The B mixture contained the same plus 1.15g L-tryptophan. The amino acids were in the

same proportion as in milk except that aspartic acid and glutamic acid were omitted because of concern about their toxicity at high doses. As they are not essential amino acids, omitting them should not have influenced the efficacy of the T- mixture in reducing tryptophan levels.

Subjects arrived in the laboratory at 8:00 am after an overnight fast. They had the purpose of the study explained to them, signed a consent form, had a baseline blood sample taken and ingested an amino acid mixture orally as a suspension as described previously (Young et al., 1985). For the next five hours they were kept in a room and entertained by being shown a movie and allowed to read magazines. The 5 hour interval is necessary for plasma tryptophan to fall to its lowest level (Young et al., 1985). At the end of this waiting period a second blood sample was taken. Then they were led into a room and allowed to select their lunch from a buffet. The foods presented and their nutritional values taken from food tables are given in Table 1. The menu was identical on each visit. For each type of food, enough was given so that some always remained at the end. In this way all the types of food were always available for selection. All the food was pre-weighed. Food intake was determined by weighing it again at the end of the eating period. Tap water was supplied with the meal. Subjects were by themselves in the room and were allowed 30 minutes to eat. To diminish any effects of cognitive function or food selection, the subjects were told that the purpose of the study was to examine the effect of food intake on mood. To reinforce this they were given various paper and pencil tests to fill in before ingesting the amino acid mixtures and before and after lunch. They were unaware that their food intake was determined. At the end of the second visit they were told of the real

purpose of the study and paid for their participation. The protocol was approved by the Ethics Committee of the Department of Psychiatry, McGill University.

The blood samples were used for the determination of plasma tryptophan, by the fluorometric method of Denckla and Dewey (1967). Large neutral amino acids in plasma were determined by high performance liquid chromatography from a subset of six patients chosen at random.

Differences between food selection and amino acid levels after ingestion of T- and B amino acid mixtures were analysed using a two tailed paired t-test.

RESULTS

Table 2 gives plasma levels of tryptophan and the other large neutral amino acids both before and after T- and B mixtures. Values for plasma tryptophan after the T- amino acid mixture were only 19% of those after the B amino acid mixture. On the other hand a comparison of the other large neutral amino acid levels after the two mixtures revealed no significant differences.

Figure 1 shows the mean protein, carbohydrate, fat and kcal taken in by the subjects after the T- or B mixtures. There was a small (14%) but statistically significant decline in protein intake after the T- mixture compared to the control B mixture. There was no significant difference in carbohydrate, fat or kcal intake. There was also no significant change in the ratio of protein intake to carbohydrate intake ($t=0.98$, $df=21$, NS).

DISCUSSION

The T- mixture caused a substantial decline in plasma tryptophan

levels. In rats a decline in plasma tryptophan of the same order of magnitude as that seen in our subjects was associated with a decline in brain 5HT of about 50% (Biggio et al., 1974). The roles of different mechanisms in producing this decline in 5HT have been studied (Gessa et al., 1974). The decline in plasma tryptophan seems to occur because the tryptophan-deficient amino acid mixture, like any mixture of essential amino acids, promotes synthesis of new protein. The tryptophan that is incorporated into this protein comes from free tryptophan in blood and tissues, resulting in a decline in its level in blood and brain. Competition at the blood brain barrier might contribute to the decline in the brain (but not blood) tryptophan. However, administration of a mixture of six amino acids which share a common transport system with tryptophan lowered rat brain tryptophan and 5-hydroxyindoleacetic acid about 20% without affecting brain 5HT. A mixture of nine of the ten essential amino acids (tryptophan is the tenth) caused larger declines in brain tryptophan and 5-hydroxyindoleacetic acid and a significant fall in brain 5HT (Gessa et al, 1974). Thus, competition for transport at the blood brain barrier plays only a small role in regulating brain tryptophan and 5HT after administration of a T- amino acid mixture. In the present study, plasma levels of the other large neutral amino acids were not significantly different five hours after administration of either T- and B mixtures (Table 2). Therefore competition at the blood brain barrier would have been the same for the two treatments, and plasma tryptophan levels, which were one fifth the value after T- mixtures compared to B mixtures, should have provided a good index of the decline in brain tryptophan.

The most likely mechanism for the decline in protein selection is a

decline in brain 5HT function. Although it is not possible to demonstrate that the fall in plasma tryptophan that we saw is accompanied by a decrease in 5HT metabolism in human brain, it would be surprising if an effect of the magnitude that we saw in plasma did not influence brain 5HT. Also, the decline in protein selection is consistent with the known effects of decreasing 5HT function in animals (Anderson, 1979; Blundell, 1984; Wurtman and Wurtman, 1984). None of the studies in which tryptophan was given to human subjects found any specific effect on protein selection (Wurtman et al., 1981; Strain et al., 1985; Hrboticky et al., 1985; Leathwood and Pollet, 1983; Blundell and Hill, in press) and the question arises as to why our results differ in this respect. Food selection is exquisitely sensitive to details of experimental design and can be influenced greatly by factors such as the degree of hunger of the subjects, the variety of food available for selection and its sensory qualities (Blundell, 1984). It is interesting from this point of view to compare our study with the one most similar in design, that of Hrboticky et al. (1985). In the latter study acute tryptophan administration decreased total calorie intake in normal male subjects. We found no increase in calorie intake with tryptophan depletion. However, this is not surprising as work with experimental animals has shown that an increase in food intake due to inhibition of 5HT metabolism is a much weaker phenomenon than the inhibition of food intake by increased 5HT function (Blundell, 1984). The lack of effect on food selection in the study of Hrboticky et al. (1985) might be explained in part by the slightly smaller variety of foods available for selection than in the present study. Cognitive, social and mood factors might also have played some role. In the study of Hrboticky et al. (1985) the subjects ate

together and were aware that food selection was being measured. In the present study the subjects ate by themselves and were not aware that food selection was being measured. Furthermore, while tryptophan administration can in some circumstances elevate mood (Young, 1986) tryptophan depletion causes a lowering of mood (Young et al., 1985). It is not known how mood might influence food selection.

While Blundell and Hill (in press) found an effect of tryptophan administration on carbohydrate selection, we found that tryptophan depletion influences selection of protein. However, the importance of precise experimental conditions in determining results of food selection experiments is seen in the report of Blundell and Hill (in press). They found tryptophan decreased carbohydrate selection when it was given with a high protein meal, but not when it was given with a high carbohydrate meal. The design of the present experiment differs from that of Blundell and Hill (in press) in several respects. Furthermore, there is no discrepancy between the findings that tryptophan supplementation can decrease carbohydrate selection while tryptophan depletion decreases protein intake, if tryptophan availability influences the relative intakes of protein and carbohydrate rather than affecting selection of either macronutrient separately. In our study the type of meal given to the subjects might have influenced the results. Thus, for many of the subjects a significant portion of their carbohydrate intake was in the form of bread. Bread is often eaten as a sandwich with high protein fillings such as ham or cheese. It is impossible to say how factors such as this could have influenced our results. Nonetheless it is quite possible that with a different series of foods to select from we might have seen an increase in carbohydrate selection rather than a decrease

protein selection after tryptophan depletion. Thus, our study does not contradict the other studies on the effect of altered tryptophan levels on food selection in humans. It merely adds to the overall picture of the types of effects that can be seen under different circumstances.

Intake of a balanced meal is associated with a modest decline in CSF tryptophan and 5-hydroxyindoleacetic acid in humans. This has been taken as evidence that a balanced meal lowers CNS 5HT in humans (PerezCruet et al., 1974). Our results raise the question of whether this decline in 5HT would be enough to alter food selection at the next meal. At the moment there are insufficient data to answer this question. Thus, although our results indicate that lowered 5HT can decrease protein selection in humans it remains to be seen whether this mechanism operates in physiological circumstances.

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Table 1. Foods available for selection and their macronutrient content

Food	Amount served	Approximate weight served (g)	kcal/g	Nutrients (g/g food)		
				Protein	Carbohydrate	Fat
White bread	12 slices	350	2.71	0.09	0.50	0.03
Butter	2 tbls.	50	7.10	0.01	0.01	0.81
Ham	10 slices	400	2.33	0.19	0	0.17
Salami	10 slices	300	3.14	0.18	0.01	0.26
Cheddar cheese	10 slices	200	3.98	0.25	0.02	0.32
Brick cheese	10 slices	200	3.71	0.22	0.02	0.30
Tomato	2 (sliced)	300	0.20	0.01	0.04	0
Apple	2 (quartered)	300	0.55	0	0.14	0.01
Coconut cookies	8	100	4.24	0.06	0.64	0.25
Chocolate chip cookies	8	125	4.71	0.06	0.70	0.21

Table 2. Plasma levels of tryptophan and the other large neutral amino acids before and five hours after ingestion of tryptophan free or nutritionally balanced amino acid mixtures

		Tryptophan free amino acid mixture		Balanced amino acid mixture	
	n	Before	After	Before	After
Tryptophan	22	70.8±11.6	20.8± 8.3	74.9±13.6	112±96
Histidine	6	114 ±25	137±36	120±19	115±26
Isoleucine	6	72.2± 7.4	189±90	86.9±12.2	143±78
Leucine	6	146 ±20	369±83	150±17	269±129
Methionine	6	32.4± 4.4	46.0±13.7	34.2± 8.2	47.4±12.6
Phenylalanine	6	72.8± 7.5	95.5±37	68.9±12.1	81.2±21.8
Valine	6	237±20	397±107	237±47	416±126

Results are given as mean ± SD in μmoles/l. A comparison of values after tryptophan free or balanced amino acid mixtures by the paired t-test revealed that values from tryptophan were significantly lower ($p<0.001$) after the tryptophan free mixture than after the balanced mixture. Values after the two amino acid mixtures were not significantly different for the other amino acids.

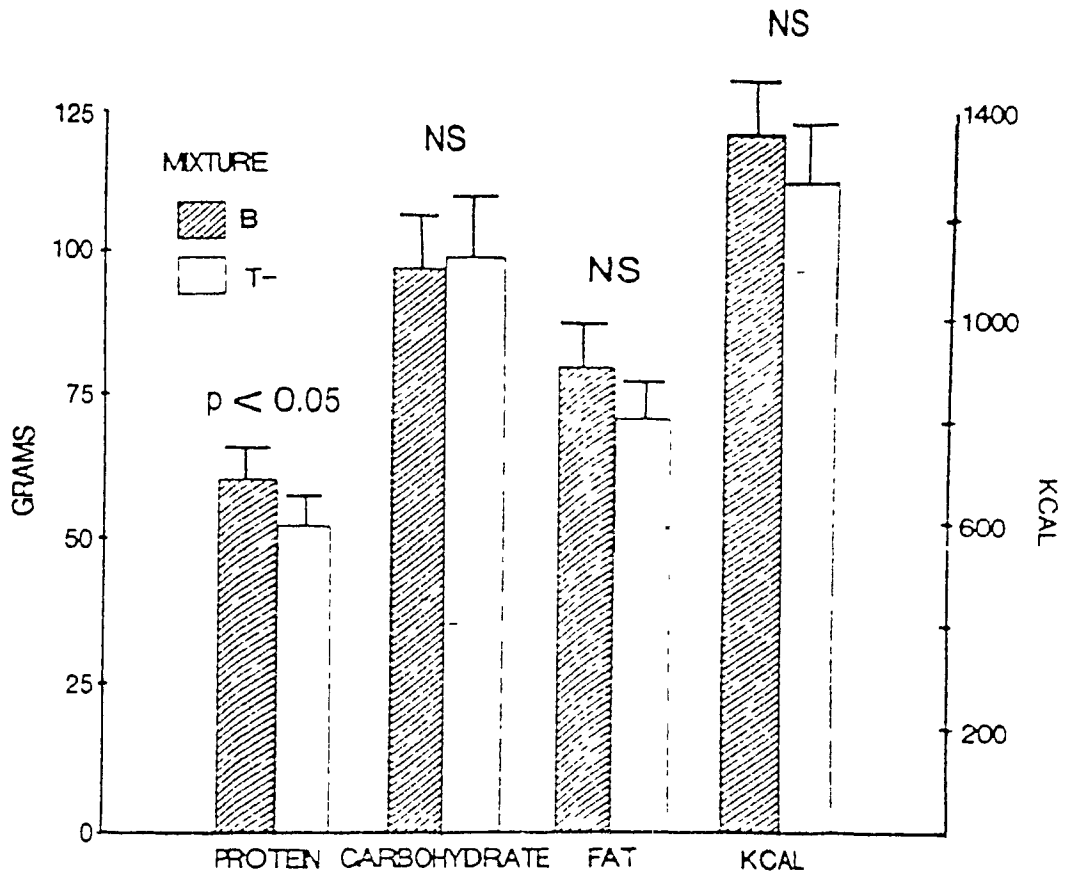

Figure 1. Intake of protein, carbohydrate, fat and kcal after balanced and tryptophan deficient (T-) mixtures. A two-tailed paired t-test showed that the T- mixture significantly decreased selection of protein ($t=2.4$, $df=21$, $p<0.05$), but not of carbohydrate ($t=-0.55$), fat ($t=1.66$) or kcal ($t=1.44$) relative to the B mixture.

PREFACE TO CHAPTER SIX

The experiemnts in this chapter are concerned with the effect of physiological amounts of protein and carbohydrate on 5HT metabolism and function in humans. The previous chapter demonstrated that lowering plasma tryptophan could alter 5HT function, as monitored by a change in a behavior thought to be mediated by 5HT, i.e. macronutrient selection. The degree of decline in plasma tryptophan after administration of the amino acid mixture used in chapter five is much greater than would be observed after protein intake. Therefore, it was necessary to determine if the ingestion of a normal quantity of either protein or carbohydrate could also alter food selection, i.e. can normal dietary constituents bring about a functional change in humans as monitored by food selection?

In the second study of this chapter, the plasma tryptophan ratio was used as an index of tryptophan availability, and therefore as an indirect, peripheral measurement of 5HT metabolism. Several reports in the literature had implicated the behavioral consequences of ingestion of high carbohydrate meals. Therefore, breakfasts containing varying proportions of protein and carbohydrate were administered to determine the relative ratio of carbohydrate to protein necessary to elicit the reported rise in the tryptophan ratio after carbohydrate. As well, a breakfast consisting of a danish pastry and coffee were given to determine, if this, a common breakfast, would increase the tryptophan ratio. It was also important to establish whether the changes observed in the tryptophan ratio were of sufficient magnitude to alter central 5HT metabolism.

Chapter 6

THE EFFECT OF PROTEIN OR CARBOHYDRATE BREAKFASTS ON SUBSEQUENT [illegible] AMINO ACID LEVELS, SATIETY, AND NUTRIENT SELECTION IN NORMAL MALES.

ABSTRACT

Normal subjects were fed protein or carbohydrate breakfasts. Both meals were in the form of a chocolate pudding and had similar sensory qualities. At lunchtime subjects were allowed to select from a buffet. The protein breakfast had a greater satiating power than the carbohydrate breakfast, but there was no difference in overall selection of protein or carbohydrate at lunchtime. However, the carbohydrate breakfast did decrease selection of apple, the only pure carbohydrate food available at lunchtime. In a second experiment changes in plasma amino acid levels were studied after subjects received carbohydrate breakfasts containing 0, 4, 8 or 12% protein, or a danish pastry. Only the 0% protein breakfast increased tryptophan availability to the brain. These experiments were performed to test the hypothesis that alterations in brain 5-hydroxytryptamine, brought about by alterations in brain tryptophan, regulate selection of protein and carbohydrate. They suggest that this mechanism does not operate in human males under normal circumstances.

INTRODUCTION

The neurotransmitter, 5-hydroxytryptamine (5HT), is a factor in the regulation of food intake. Generally, pharmacological and lesion studies in rodents have shown that depleting brain 5HT results in hyperphagia, while increases in brain 5HT have an anorectic effect (for a review see, Blundell, 1984). More recently it has been demonstrated that animals, in addition to regulating their total kilocalorie intake, can maintain the proportion of protein and carbohydrate in their diet on a meal-to-meal basis (Musten et al., 1974). Some experiments have shown that diet manipulations can alter relative protein and carbohydrate [illegible], and there is also some evidence that dietary protein and [illegible] themselves alter brain tryptophan and 5HT. An hypothesis [illegible] based on the biochemical relationship between tryptophan, [illegible] macronutrient intake (Anderson, 1979; Wurtman and Wurtman, 1984; [illegible], 1984).

To enter the brain, tryptophan must compete with other large neutral amino acids for transport by a common carrier system (Oldendorf and Szabo, 1976). Therefore, it is the plasma ratio of tryptophan to the sum of its competitors which becomes the determining factor of tryptophan availability to the brain. The dietary macronutrients protein and carbohydrate affect the ratio in opposite ways. Protein, which contains a high concentration of competitors relative to tryptophan can, in some circumstances, lower the plasma ratio, and subsequently brain tryptophan (Glaeser et al., 1983). In contrast, carbohydrate increases brain 5HT. Insulin, released after carbohydrate ingestion causes uptake of the branched-chain amino acids into muscle. The concentration of the competitors is then lowered, allowing more tryptophan to enter the brain and increasing brain 5HT (Fernstrom and Wurtman, 1971). It has been argued that the raising or lowering of brain 5HT will initiate

compensatory mechanisms to alter macronutrient selection (Wurtman and Wurtman, 1977; Anderson, 1979). For example, after a protein meal, the decline in brain tryptophan (Glaeser et al, 1983) may lead to a decline in brain 5HT. The decrease in brain 5HT is presumed to cause an increase in selection of carbohydrate relative to protein at the next meal, which, to complete the cycle, should alter brain 5HT. Conversely, carbohydrate raises brain 5HT, which will decrease subsequent selection of carbohydrate relative to protein. In this way the intake of macronutrients could be regulated within certain limits (see Wurtman and Wurtman (1986) for a review of this position).

In the rat, pharmacological manipulations of 5HT are consistent with this hypothesis (Wurtman and Wurtman, 1979; Ashley et al., 1979) but dietary treatments have given both positive (Ashley and Anderson, 1975; Wurtman et al., 1983) and negative (Peters and Harper, 1984) results.

Few human experiments examining the role of 5HT on food selection have been undertaken. Hrbotticky et al. (1985) examined the effect of 3 different doses of tryptophan. Energy intake was reduced with 2 and 3 g of tryptophan, but no effect was observed on the proportion of carbohydrate or protein selected. Tryptophan (2 g) was also used in subjects with a propensity for carbohydrate snacks (Wurtman and Wurtman, 1983), though no significant effects were observed. Strain et al. (1985) administered 1 g of tryptophan, with 10 g of carbohydrate, 3 times a day for 3 months to obese subjects. Again no significant effects were observed. However, Blundell and Hill (1987) studied the effect of giving 1 g of tryptophan along with a high protein or high carbohydrate lunch. They found that tryptophan decreased carbohydrate selection when given with the high protein meal (Blundell and Hill, 1987). These studies

emphasize the sensitivity of results in food selection experiments to the procedural aspects of experimental design.

In an earlier study we examined the effect of lowering plasma tryptophan on food selection in normal males (Young et al., in press). A tryptophan deficient amino acid mixture was administered orally, reducing plasma tryptophan by 81%. Five hours later, when plasma tryptophan was at its lowest, the subjects were allowed to select from a buffet lunch. We found a small but significant decline in protein intake associated with tryptophan depletion. This suggests that a decrease in brain 5HT can alter protein selection in humans. This is consistent with the presumed effect and mode of action of the prior ingestion of a high protein meal. However, the effect of tryptophan depletion on brain tryptophan will be much greater than that of protein ingestion, and there is no direct evidence that a similar effect would be obtained with consumption of a high protein meal. Therefore, we have performed two experiments designed to examine how the amount of protein consumed in a meal influences subsequent food selection and plasma tryptophan ratios. The outcome of these studies should disclose whether the effects of protein intake upon subsequent nutrient selection are mediated via changes in the plasma ratio of tryptophan to the sum of the other large neutral amino acids and therefore via changes in brain tryptophan and 5HT.

MATERIALS AND METHODS

Subjects

The subjects were normal males between the ages of 18 and 30 who were recruited through newspaper advertisements. All had at least a high school education, no history of psychiatric disorders or food allergies,

and were within 10% of their ideal body weight for their height. They were not taking any prescription medication and found the foods in the study acceptable. Each subject signed an informed consent form concerning the nature of the experiment. The protocol was approved by the Ethics committee of the Department of Psychiatry, McGill University. In experiment 1, 32 subjects took part and in experiment 2 there were 50 subjects.

Diets

In experiment 1 subjects received either a protein or a carbohydrate meal in the form of a chocolate pudding. The protein pudding consisted of three 9 oz. packets (mixed with 210 ml. of water) of a protein supplement manufactured by Bariatrix International Inc. of Montreal. Each pudding contained 45g of protein, 12g of carbohydrate and approximately 3g of fat. The total caloric content was 210 Kcal. The carbohydrate pudding was made up in our laboratory. It consisted of 40g of cornstarch, 28g of Hershey's chocolate syrup and 53g of polycose, a non-sweet caloric supplement from Ross laboratories., totaling 100g of carbohydrate and approximately 400 Kcal. It was prepared by adding 210 mls of water to the cornstarch and cooking over boiling water until thick. The polycose and the chocolate syrup were then added slowly to the mixture. The carbohydrate meal contained more calories than the protein meal because a normal diet contains more carbohydrate than protein. Before the actual experiment, puddings were rated with respect to taste, appearance and texture by 15 students. Subjects were required to taste both puddings, rate them on a five point scale for taste, texture and appearance and guess their nutrient composition. No significant differences were found between the two puddings on any of the

variables tested and the guesses of the macronutrient composition were not significantly different for the two puddings. An important methodological feature of this study was to equate the physical state of the protein and carbohydrate meals so as to prevent the subjects from responding in accordance with the familiar distinction between the savory and sweet aspects of these nutrients. Thus, differences in response to the two meals could be attributed to the different metabolic effects of the nutrients and not to any taste sensation or cognitive attributions.

In experiment 2, subjects received one of five different meals. Four of the treatments consisted of isocaloric (400 Kcal) chocolate puddings with protein contents of 0, 4, 8, and 12%. The carbohydrate pudding (0% protein) was the same as in experiment 1. Protein content was adjusted by mixing in appropriate amounts of the Dietetic chocolate pudding mix, while equivalent amounts of carbohydrate pudding were removed. To simulate a common North American breakfast, the fifth treatment consisted of a cherry danish pastry (purchased from Steinberg's Inc.) and 1 cup of coffee. The pastry contained 46 g carbohydrate, 5 g protein, 16 g fat and 342 kcal.

Experimental Procedure

In experiment 1 a double-blind counterbalanced crossover design was used. Each subject came into the laboratory on two days, one week apart. On one occasion they were given the protein pudding and on the other day the carbohydrate pudding. Seventeen of the subjects were given the protein pudding first, while fifteen received the carbohydrate pudding first. Subjects arrived in the laboratory at 8:00 a.m. after an overnight fast. They were told the purpose of the study was to investigate the behavioral effects of nutrients and were required to fill

out various psychological tests during the course of the study. They were not told of the dietary composition of the pudding, though they were told it was composed of normal dietary constituents. At 9:00 a.m., they were required to eat the entire chocolate pudding after which they were permitted to read and watch a movie provided. They were not allowed to leave the laboratory. Three hours later, the subjects were individually brought into a room and allowed to select their lunch from a buffet. The foods offered were the same that we used previously (Young et al., 1988) and their nutritional values are shown in Table 1. The menu was identical on each visit. For each type of food, enough was given so that some always remained at the end and therefore was always available for selection. Subjects ate alone in the room for 30 minutes. All food and water were previously weighed. Food intake was determined by weighing what was left at the end of the meal and subtracting from the original weight. Subjects were unaware that their food intake was being monitored. At the end of the second trial, they were told of the real purpose of the study and paid for their participation.

In experiment 2 each subject came into the laboratory at 8:30 a.m. after an overnight fast and was randomly assigned to one of the five treatment groups with 10 subjects in each group. After completion of the questionnaires, a 10 ml venous blood sample was taken. At 9:00 a.m. they were required to eat either the entire chocolate pudding or danish pastry, after which they were permitted to read and watch a movie provided. Two hrs later at 11:00 a.m., 20 ml of blood was drawn again. Two food questionnaires were given, before 9:00 a.m. and at 10:45 a.m. The first, a forced choice, contained a list of 16 food pairs, one high protein and one high in carbohydrate. The subjects were required to

select which of each pair they would prefer. The measure used was the total number of high protein or high carbohydrate foods selected. The second questionnaire consisted of a list of 32 foods together with portion sizes. The subjects were asked to indicate the foods they would prefer to eat in a meal at that time. The macronutrient composition of the meal was calculated. This method provides a more convenient way of looking at food selection than the real food selection performed in the first experiment. These procedures have previously been shown to be sensitive to nutrient manipulations (Blundell and Hill, 1987).

Analytical Methods

Blood was collected into heparinized evacuated tubes and centrifuged immediately. The plasma was used for the determination of free and total plasma tryptophan as well as the plasma levels of the other large neutral amino acids. The free plasma tryptophan concentration was taken as the concentration in an ultrafiltrate of plasma prepared at 25°C in an Amicon MPS-1 centrifugal ultrafilter using YMT membranes. Tryptophan in the ultrafiltrate and in the plasma were measured by the fluorometric method of Denckla and Dewey (1967). Plasma was prepared for amino acid analysis by adding 50 mg of 5-sulfosalicylic acid to 1 ml of plasma. Samples were vortexed, left for 1 hr. at 4°C and centrifuged at 6000 rpm for 10 min. The supernatant was mixed with 0.3N Lithium Hydroxide in a ratio of 2.5:1.0, then assayed on an LKB Alpha Plus amino acid analyzer.

Statistical Analysis

The effect of protein and carbohydrate meals on total Kcal, macronutrient selection and individual foods selected was assessed using a two-tailed paired t-test. The effect of varying protein content on

plasma amino acids was analyzed using a 2-way ANOVA. Comparisons of selected means were made using Tukey's test. A probability of 5% was taken as the level of statistical significance.

RESULTS

Experiment 1

Fig. 1 shows differences in total kilocalorie intake with respect to previous carbohydrate or protein intake, day of trial, and the effect of each macronutrient depending on the order of administration. There was no significant difference in the amounts of food consumed in the meals following the high protein or high carbohydrate breakfasts, although slightly fewer calories were consumed after the protein. However, since the carbohydrate breakfast (400 kcal) was almost double the energy value of the protein breakfast (210 kcal) it is clear that calorie for calorie the protein breakfast had a much greater satiating effect. In addition, a significant decrease in total kilocalorie intake was observed on the second day taking both breakfasts together. Due to this order effect, the group of subjects was broken down into those who had received protein first and those who had received carbohydrate first. Although no differences were found in the energy intake in the group which had received protein first, there was a significant decrease in kilocalorie intake in the group that had received carbohydrate first. Thus, when protein was given as the second treatment, even though it contained about half the calories of the carbohydrate breakfast, there was a significant decrease in the absolute number of calories consumed in the test meal, indicating a very potent satiating action.

Figs. 2 and 3 show protein and carbohydrate intake with respect to previous carbohydrate or protein intake, day of trial and the effect of

each macronutrient depending on the order of administration. Overall there was no effect of the different premeals on subsequent selection of protein or carbohydrate. Subjects selected significantly less protein on the second day compared with the first day. There was a similar trend for carbohydrate, but it was not statistically significant. The groups were broken down into those who had received protein first and those who had received carbohydrate first. This revealed that the decline in total kilocalorie intake after protein, which occurred only in the subjects who received protein second, was due to declines in both protein and carbohydrate intake. The declines in total kcal, protein and carbohydrate were 16%, 15.9% and 15.6% respectively, although the decline just failed to reach statistical significance for the protein intake. Once again, since the effects of the protein breakfast were achieved with approximately half the energy value of the carbohydrate breakfast, the protein is clearly exerting a more potent suppressive action on the subsequent intake of both macronutrients. In Table 2, the amount of each food selected after both treatments is shown. The trend for the protein breakfast to decrease subsequent food intake is seen for many of the individual foods. However, the only significant finding is in the opposite direction to this trend. Thus, significantly less apple was selected after the carbohydrate pudding than after the protein pudding. The apple was the only item available for selection which consisted almost entirely of a single macronutrient suggesting that this seemingly inconsequential result may have had biological significance. This is dealt with in greater detail in the discussion.

Experiment 2

Meals taken at breakfast which differ only by 20% in their protein

content lead to differences in the plasma tryptophan ratio (Ashley et al., 1985). Therefore, the protein and carbohydrate breakfasts in Experiment 1 would have led to differences in the plasma tryptophan ratio. These biochemical differences are accompanied by one main behavioral difference. The protein breakfast resulted in a greater satiating effect (taking into account the calories ingested at breakfast) than the carbohydrate breakfast. However, this effect was seen equally on protein and carbohydrate intake at lunch. The differences in breakfast macronutrient composition did not lead to altered macronutrient selection at lunch. Thus, our data are not consistent with one part of the hypothesis which relates food-induced alterations in brain 5HT to subsequent macronutrient selection. Experiment 2 was designed to look at another of the links in this hypothesis. Foods differing greatly in macronutrient will result in differences in the plasma tryptophan ratio. We wished to study how large differences in the ratio of protein to carbohydrate in a meal must be in order to result in significant differences in the plasma tryptophan ratio. We also studied the effect of different breakfasts on changes in subsequent intended food consumption. However, because this was not the primary purpose of the experiment, and because of the number of conditions in the study, nutrient preference was not measured directly, as in experiment 1, but indirectly by checklists and forced choice procedures.

In experiment 2 we looked at plasma amino acids after isocaloric carbohydrate breakfasts containing 0, 4, 8 and 12% protein. Mean plasma levels of all the large neutral amino acids declined after the pure carbohydrate and 4% protein treatment, though the declines were smaller in the 4% group (Table 3). At 8 and 12% protein none of the changes were

statistically significant. Tryptophan and histidine exhibited the smallest decline after carbohydrate. This particular effect, along with the larger decrease in the other neutral amino acids, resulted in a significant increase in the ratio of tryptophan to the sum of the competing amino acids after a carbohydrate breakfast (Table 4). There was an increase in the histidine ratio of the same magnitude, but it failed to achieve statistical significance. The addition of as little as 4% protein to the carbohydrate breakfast prevented the increase in the tryptophan ratio. The 4, 8 and 12% protein breakfasts and the danish pastry all failed to influence any of the amino acid ratios significantly.

In Table 4a, the ratio was calculated using the traditional five amino acids; tryptophan, valine, leucine, isoleucine, phenylalanine and tyrosine. The ratios in Table 4b were calculated using the same five amino acids but histidine and methionine were included. These two amino acids are also competitors at the blood brain barrier (Oldendorf and Szabo, 1976). When the ratios were calculated in this manner, the tryptophan ratio did not quite reach significance. Results of the food selection and forced choice questionaires are shown in Table 5. No statistically significant effects were observed, although there is a trend towards decreasing protein selection as the protein content of the breakfast increased.

DISCUSSION

The first experiment was designed to determine if physiological amounts of the macronutrients could alter nutrient selection at the following meal. We required treatments that would approximate proportions and quantities of macronutrients normally ingested. As most

meals generally contain twice as much carbohydrate as protein, we decided on a non-isocaloric approach. The protein treatment contained slightly less than half the amount of kilocalories as the carbohydrate treatment. The amount of protein we used, 45 g, is a moderate quantity, and might be ingested in a single meal. Protein has been shown to possess a greater satiating capacity than carbohydrate in man (Blundell and Hill, 1987) and monkeys (Jen et al., 1985). Thus, to be able to observe an effect on nutrient selection without altering total kilocalorie intake, it seemed appropriate to administer less protein. This approach was justified by the absence of any difference between treatments on total kilocalorie intake as seen in Fig. 1. We did find that a protein meal decreased subsequent energy intake when it was given on the second occasion, but not when it was given on the first occasion. Psychological factors may have played a part in the order effect. On the second day not only were the foods familiar and thus possibly less appealing, but the subjects were anxious to be paid and to leave. These factors may have combined with the susceptibility of protein to suppress subsequent food intake and caused the order effect that we found.

Our results indicate that high protein and high carbohydrate meals given in the morning displayed different satiating capacities. A high protein breakfast, with half the energy value of a high carbohydrate breakfast, exerted an approximately equipotent effect on a lunchtime test meal. Since the protein and carbohydrate breakfasts were similar in sensory qualities, and therefore presumably similar in their capacity to provoke cognitive attributions, it is a reasonable assumption that their different actions on satiety were mediated by different metabolic effects. The exact metabolic substrate of this effect is unknown, but

our data indicate that it is not 5HT. If the carbohydrate breakfast raised, and the protein breakfast lowered brain 5HT, then a difference should have been seen at lunchtime in the absolute number of calories selected. In fact the number of calories selected was approximately the same after the two breakfasts and the greater satiating power of the protein breakfast could only be inferred from the fact that intake was the same after the protein breakfast even though fewer calories were ingested.

The lack of effect of the different breakfasts on energy intake is not surprising as human studies designed to investigate the acute role of 5HT in regulation of energy intake have shown mixed results. Although tryptophan administration led to a decrease in food intake in young men (Hrboticky et al., 1986), the finding is confounded by the increased faintness and dizziness experienced by the treated subjects. Blundell and Hill (1987) found that adding tryptophan to either a high carbohydrate or a high protein lunch failed to influence total calorie intake in a test meal three hours later. We also failed to detect any effect on total calorie selection in a test meal when tryptophan levels were altered. In our experiment plasma tryptophan was depleted markedly by a tryptophan-deficient amino acid mixture (Young et al., in press).

In the present study we did not find any changes in macronutrient selection after pretreatment with either protein or carbohydrate (Fig. 1). This is in contrast to the results of Blundell and Hill (1987) who found a significant decrease in protein selection after a high protein meal. However, Blundell and Hill (1987) used 66 g of protein, nearly 50% more than we used (45 g). It appears that the relatively small quantities of protein used in our study were not enough to alter

macronutrient intake. Alternatively the difference in energy intake at breakfast may have been a confounding factor. It may be that altered macronutrient selection would have been seen if the caloric intake of the protein and carbohydrate breakfasts had been equal. The lack of any effect should not be surprising as studies on the effect of altered tryptophan levels on macronutrient selection in humans, like those on total energy intake, have given mixed results. Changes in macronutrient selection after tryptophan administration have not been observed in some studies (Hrboticky et al., 1985; Wurtman and Wurtman, 1981; Strain et al., 1985). However, when tryptophan was given with a high protein lunch, a significant reduction in carbohydrate selection was seen (Blundell and Hill, 1987). Conversely, protein intake was diminished in subjects whose plasma tryptophan levels were lowered by a tryptophan deficient amino acid mixture (Young et al., in press). The results of these studies, together with those of the present study, suggest that 5HT can play a role in macronutrient selection in some circumstances, but that diet-induced changes in brain 5HT are unlikely to play an important role in regulating intake of protein and carbohydrate in humans.

Comparison of individual foods selected after macronutrient administration revealed a significant increase in apple consumption after protein pretreatment (Table 2). This seemingly inconsequential result led us to examine the relative amounts of protein and carbohydrate in the foods offered and to question why the effect was specific to this particular food. When the ratios of carbohydrate to protein were calculated we found the ratio for the apple was noticeably different from that of the other foods in that it was more than 5-fold higher than the next highest ratio (Table 2). It was the single food containing

primarily carbohydrate, with only negligable amounts of protein. In contrast, the other "carbohydrate" foods contained more that 4% protein energy. A protein content of 4% is significant because, as discussed below, a protein content that small is capable of blocking the rise in the tryptophan ratio produced by carbohydrate. Therefore, as far as the plasma ratio is concerned, only the apple can be classified as a carbohydrate food. In terms of tryptophan availability, all the other foods would be perceived neurochemically as mixed carbohydrate and protein foods, even the tomato and cookies. The fact that a carbohydrate meal decreased selection of the only relatively pure carbohydrate food in the subsequent test meal suggests that this small finding did not occur only by chance. It may be that in humans the mechanisms altered by previous protein or carbohydrate meals are not involved so much in regulating overall protein or carbohydrate intake as in the regulation of intake of specific carbohydrate foods. This makes sense in evolutionary terms. Fruits such as mangoes are highly preferred by monkeys. A mechanism would be needed to stop monkeys eating nothing but mangoes during mango season, because this would result in negligible protein intake. Our results suggest that the intake of a meal of mangoes might inhibit only the intake of further items containing high carbohydrate with little or no protein.

Because of the considerations above we decided to look at the amount of protein in a carbohydrate meal necessary to block the rise in the tryptophan ratio. In addition we looked at a commonly ingested breakfast, coffee and a danish pastry, that is generally regarded as a carbohydrate breakfast. In fact, in such a breakfast the amount of protein is about 10% the amount of carbohydrate. Previously published

reports had only looked at the effect of larger amounts (20% or over) of protein or pure carbohydrate (Ashley et al., 1982; Fernstrom et al., 1979). The effect of smaller quantities of protein on human plasma amino acids had not been examined. The results from our second study supported the idea that only relatively pure carbohydrate significantly elevated the tryptophan ratio. We found that the addition of only 4% protein was sufficient to inhibit the increase (Table 4). Beginning at the 8% protein level, a trend towards a decrease in the tryptophan ratio was seen, increasing with the increased protein content of the meal. The breakfast of a coffee and a danish pastry caused no significant change in the tryptophan ratio, indicating that it was not a carbohydrate meal as far as the tryptophan ratio is concerned.

Interestingly, the ratio of histidine to the sum of the neutral amino acids showed the same response as tryptophan, with an increase of about 25% after the carbohydrate treatment. This amino acid precursor has been virtually ignored in the behavioral studies, even though it is able to compete at the blood brain barrier with the other neutral amino acids and the rate-limiting enzyme is also unsaturated at physiological concentrations of substrate (Schwartz et al., 1970).

A trend towards an inverse relationship between protein content and protein selection was seen in the food selection and forced choice questionnaires (Table 5), though these differences did not reach statistical significance.

Our human data concerning the effects of small amounts of protein, added to a carbohydrate meal, on the plasma tryptophan ratio is supported by the animal work of Yokogoshi and Wurtman (1986) who found that 5% casein added to a 70% carbohydrate meal blocked the expected increase in

the tryptophan ratio. Therefore in both rats and man, significant changes in the ratios will occur only at extreme levels of macronutrient ingestion, i.e. when either pure carbohydrate or high concentrations of protein are consumed. This supports our contention that the tryptophan ratio may be involved in a mechanism used to distinguish between foods containing pure macronutrients. A free ranging animal would perhaps be in a situation where foods of extreme macronutrient content exist and would be forced to select among them. Upon examination, very few foods actually contain less than 4% protein. The foods falling into this category would be primarily fruit, which are usually associated with a sweet taste. For animals in the wild, the purpose of the mechanism may be to inhibit an animal from feeding solely on a food which is preferable for its sensory rather than its nutritional quality.

In humans, the physiological relevance of this mechanism is questionable, as most meals contain a mixture of protein, carbohydrate and fat in quantities unlikely to increase the tryptophan ratio significantly. A second factor which must be considered is the magnitude of change found after the carbohydrate breakfast. Generally, it is assumed that a 50-100% increase or a 30-50% decrease in the tryptophan ratio is required to alter brain 5HT metabolism (Ashley et al., 1985). Recently we have given protein and carbohydrate meals to patients before they had a lumbar puncture. Measurements of tryptophan and the 5HT metabolite, 5-hydroxyindoleacetic acid, in cerebrospinal fluid were consistent with the idea that changes of the plasma tryptophan ratio of the size mentioned will not lead to alterations in CNS 5HT synthesis (Teff et al, in press). In our present study, the carbohydrate meal only

resulted in an increase in the 20% range. It is unlikely that this degree of change would effect brain 5HT. Thus in human males, the evidence suggests that under normal dietary conditions the tryptophan ratio does not play an important role in the overall regulation of food intake or nutrient selection, although it is possible that in clinical populations with abnormal eating patterns or biochemistry, the mechanism is influential.

The lack of evidence for a role of the tryptophan ratio in regulation of food intake does not imply that 5HT itself is not involved in the behavior. Abundant animal data points towards 5HT as an important neurotransmitter in the regulation of food intake, but as experimental design becomes more sophisticated we are finding that these effects are very subtle. One example is the role of 5HT in the termination of meals (Blundell, 1986; Shor-Posner et al., 1986). Regulation of food intake is a highly complex behavior, especially in humans. In addition to physiological mechanisms, cultural, psychological and sensory factors combine to influence human dietary intake. It is these combined factors which make interpretation of dietary experiments so difficult and which accounts for differences observed in various dietary paradigms. It will be necessary to develop experimental paradigms for humans that are as finely structured as those existing for animals in order to determine the precise role of 5HT and its relationship to dietary intake.

In conclusion, the results of the experiments presented here indicate that high protein or high carbohydrate breakfasts do appear to exert metabolic effects which result in changes in satiety and subtle alterations in subsequent dietary selection. However, the changes in plasma tryptophan ratios indicate that these effects are not mediated by

changes in CNS tryptophan or 5HT metabolism.

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Table 1. Foods available for selection and their macronutrient content

Food	Amount served	Approximate weight served (g)	kcal/g	Nutrients (g/g food)		
				Protein	Carbohydrate	Fat
White bread	12 slices	350	2.71	0.09	0.50	0.03
Butter	2 tablespoons	50	7.10	0.01	0.01	0.81
Ham	10 slices	400	2.33	0.19	0	0.17
Salami	10 slices	300	3.14	0.18	0.01	0.26
Cheddar cheese	10 slices	200	3.98	0.25	0.02	0.32
Brick cheese	10 slices	200	3.71	0.22	0.02	0.30
Tomato	2 (sliced)	300	0.20	0.01	0.04	0
Apple	2 (quartered)	300	0.55	0	0.14	0.01
Coconut cookies	8	100	4.94	0.06	0.64	0.25
Chocolate chip cookes	8	125	4.71	0.06	0.70	0.21

Table 2. Amount of food selected at lunchtime after carbohydrate or protein breakfasts and relative macronutrient composition of foods offered.

	Amount of Foods Selected at lunchtime (g)		Relative macronutrient content of foods available for selection at lunch		
	Carbohydrate breakfast	Protein breakfast	Carbohydrate/ Protein	%Protein	%Protein Energy (kcal)
Bread	95.4 ±41.0	91.8 ±42.0	5.80	8.6	12.7
Butter	7.31± 6.71	7.78± 6.63	1.00	0.7	0.4
Ham	87.3 ±55.8	85.3 ±70.0	0.00	19	33
Salami	52.9 ±38.2	52.8 ±35.7	0.08	15	23
Cheddar Cheese	44.3 ±32.9	42.7 ±32.6	0.08	25	25
Brick Cheese	50.3 ±34.1	59.1 ±35.4	0.09	22	24
Tomato	97.9 ±78.0	91.4 ±73.0	4.00	1.1	20
Coconut cookies	8.16±13.8	5.66±11.4	10.0	6.2	5.0
Choc. chip cookies	33.1 ±29.7	25.3±31.3	12.0	5.5	4.7
Apple	70.1±82.8	10.5±87.5*	68.0	0.2	1.5

Values for the amount of foods selected are given as mean of 32 ± S.D.

*, $p<0.05$ by two-tailed paired t-test comparing amount of food selected at lunchtime after carbohydrate or protein breakfasts.

Table 3. Effect of dietary treatments on plasma amino acid levels.

Breakfast	Pre or post meal	Trp	Free Trp	Tyr	Phe	Val	Ile	Leu	His	Met
0% protein	pre	72.6±18.7	10.9±3.7	61.0±10.5	54.4± 8.9	226±60	70.2±12.3	124±49	85.5± 9.6	28.2±4.6
	post	59.6±17.2	7.7±1.8*	38.5± 8.9*	40.6±11.4	162±42**	38.7± 8.6**	82±17*	73.4±14.8	17.9±3.4**
4% protein	pre	67.0± 8.11	9.3±1.6	51.7± 8.6	50.2± 7.7	232±44	65.3±14.3	133±27	82.1±15.8	23.7±4.1
	post	56.8± 7.27	7.4±1.1	41.1± 5.8	42.3± 4.3	189±28*	46.6± 6.1*	92±11*	71.6±12.1	18.8±2.6*
8% protein	pre	72.1±12.7	10.3±2.0	56.5±13.1	50.2± 9.2	213±42	64.5±12.7	123±23	74.7±11.3	23.9±5.7
	post	65.2±13.3	8.5±1.6	58.1± 8.6	51.3± 5.1	209±24	58.9± 6.0	109±13	77.3± 9.5	24.4±3.2
12% protein	pre	65.7±10.8	9.8±1.5	54.1± 6.3	53.3± 7.0	230±49	70.7±14.4	141±86	86.5±12.9	25.4±2.7
	post	63.4±10.5	8.4±1.2	57.1± 8.2	56.1± 7.2	246±47	70.9±13.1	136±35	86.9±13.8	25.7±2.6
Danish pastry	pre	78.6±14.7	11.8±3.5	66.5±25.2	62.7±13.8	242±73	86.6±47.0	162±66	91.8±15.3	28.5±8.7
	post	67.6±14.8	9.9±2.7	60.1±25.4	63.1±15.8	219±58	68.8±31.6*	130±48	88.2± 9.7	24.1±8.3*

Results are presented as mean of 10 ± S.D. in μmoles/l. A two way analysis of variance was carried out on the values for each of the individual amino acids. There was a significant effect of time on total plasma tryptophan ($F(1,18)=8.11$, $p<0.01$) and free plasma tryptophan ($F(1,18)=17.7$, $p<0.01$). Tukey's test revealed a significant decline in free tryptophan with the 0% protein meal ($q=3.95$, $p<0.05$). For tyrosine there was a significant effect of time ($F(1,18)=7.22$, $p<0.05$) and a significant time by condition interaction ($F(4,72)=2.71$, $p<0.05$). Tukey's test revealed a significant decline in plasma tyrosine after the 0% protein breakfast ($q=5.06$, $p<0.05$). For phenylalamine there was no significant effect of time, but there was a significant time by condition interaction ($F(4,72)=2.65$, $p<0.01$). There was a significant effect of time for valine ($F(1,18)=9.99$, $p<0.01$), isoleucine ($F(1,18)=17.7$, $p<0.01$) and leucine ($F(1,18)=13.4$, $p<0.05$). Tukey's test revealed significant declines for the branched chain amino acids for the 0% breakfast (for val, $q=5.38$, $p<0.01$; for ileu, $q=5.64$, $p<0.01$; for leu, $q=4.0$, $p<0.05$) and the 4% breakfast (for val, $q=3.66$, $p<0.05$, for ileu, $q=3.33$, $p<0.05$, for leu, $q=3.88$, $p<0.05$) while the danish pastry caused a significant decline in ileu ($q=3.17$, $p<0.05$). For methionine there was a significant effect of time ($F(1,18)=16.6$, $p<0.01$) and a significant time by condition interaction ($F(4,72)=3.70$, $p<0.05$). Tukey's test revealed a significant decline in methionine after the 0% ($q=7.06$, $p<0.01$) and 4% breakfast ($q=3.36$, $p<0.05$) and after the danish pastry ($q=3.01$, $p<0.05$). *, $p<0.05$, **, $p<0.01$ relative to premeal value.

Table 4. Effect of dietary treatments on plasma amino acid ratios.

A.

Breakfast	Pre or post meal	Trp/LNAA	Free Trp/LNAA	Tyr/LNAA	Phe/LNAA	His/LNAA
0% protein	pre	0.133±0.029	0.020±0.006	0.110±0.019	0.096±0.008	0.144±0.028
	post	0.168±0.042*	0.022±0.005	0.103±0.022	0.105±0.015	0.179±0.051
4% protein	pre	0.129±0.030	0.018±0.004	0.095±0.015	0.091±0.006	0.137±0.026
	post	0.140±0.026	0.018±0.004	0.097±0.016	0.099±0.008	0.153±0.027
8% protein	pre	0.144±0.025	0.020±0.003	0.107±0.015	0.095±0.009	0.131±0.021
	post	0.135±0.029	0.018±0.003	0.118±0.015	0.103±0.009	0.141±0.022
12% protein	pre	0.122±0.025	0.018±0.004	0.099±0.019	0.097±0.049	0.142±0.021
	post	0.112±0.017	0.015±0.003	0.101±0.014	0.099±0.015	0.140±0.027
Danish pastry	pre	0.132±0.026	0.019±0.003	0.105±0.013	0.102±0.021	0.136±0.025
	post	0.129±0.025	0.019±0.003	0.107±0.016	0.118±0.023	0.152±0.024

B.

0% protein	pre	0.110±0.024	0.017±0.006	0.091±0.014	0.080±0.006	0.134±0.021
	post	0.134±0.035	0.018±0.004	0.082±0.016	0.085±0.012	0.172±0.048
4% protein	pre	0.108±0.024	0.015±0.003	0.079±0.013	0.076±0.005	0.133±0.025
	post	0.115±0.021	0.015±0.003	0.080±0.014	0.082±0.006	0.148±0.026
8% protein	pre	0.120±0.020	0.017±0.003	0.090±0.013	0.080±0.007	0.126±0.020
	post	0.111±0.024	0.015±0.003	0.098±0.011	0.086±0.007	0.135±0.021
12% protein	pre	0.101±0.020	0.015±0.003	0.082±0.014	0.081±0.014	0.137±0.020
	post	0.094±0.013	0.013±0.003	0.084±0.011	0.082±0.011	0.134±0.026
Danish pastry	pre	0.109±0.020	0.016±0.002	0.088±0.011	0.086±0.017	0.131±0.023
	post	0.106±0.019	0.015±0.002	0.089±0.014	0.097±0.019	0.146±0.029

In section A the ratios are calculated using Trp, Tyr, Phe, Leu, Ileu and Val as the competing amino acids. In section B, Met and His are also included. Values are given as mean of 10 ± S.D. For each ratio a two way analysis of variance was carried out separately for the values in sections A and B. None of the time by condition interactions achieved significance, but the values for tryptophan in section A were close to significance ($F(4,72)=2.42$: for $p=0.05$ a value of 2.48 is needed). Tukey's test revealed that only the 0% protein meal caused a significant change in the tryptophan ratio ($q=4.73$, $p<0.05$).

Table 5. Effect of dietary treatments on food selection and forced choice questionnaires

	Food Selection			
	Protein (g)		Carbohydrate (g)	
Pre meal	pre	post	pre	post
0% protein	133±63	191±102	236±114	[illegible]
4% protein	141±59	180±51	202±115	[illegible]
8% protein	125±93	146±78	222±140	[illegible]
12% protein	130±70	129±84	266±124	[illegible]
breakfast	136±107	160±108	255±165	[illegible]

	Forced Choice			
	Number of high protein foods		Number of high carbohydrate foods	
Pre meal	pre	post	pre	post
0% protein	9.0±4.8	11.6±3.6	6.9±4.7	4.4±3.6
4% protein	10.2±2.5	11.7±2.0	5.8±2.5	4.3±2.0
8% protein	7.0±5.4	8.0±3.7	9.0±5.4	7.9±3.7
12% protein	8.8±5.0	6.9±5.2	8.2±4.9	9.1±5.2
breakfast	7.5±5.0	8.0±4.8	8.5±5.0	8.0±4.9

Values for food selection show the amount of protein and carbohydrate in the foods selected by the subject from a menu before and after ingestion of a protein or carbohydrate breakfast. Values are the mean of 10±SD. Results of the forced choice questionnaire are given as mean of 10±SD for number of high protein or high carbohydrate foods selected. Two way analysis of variance revealed significant time by condition interactions, indicating that treatment effects were not different for the different treatments.

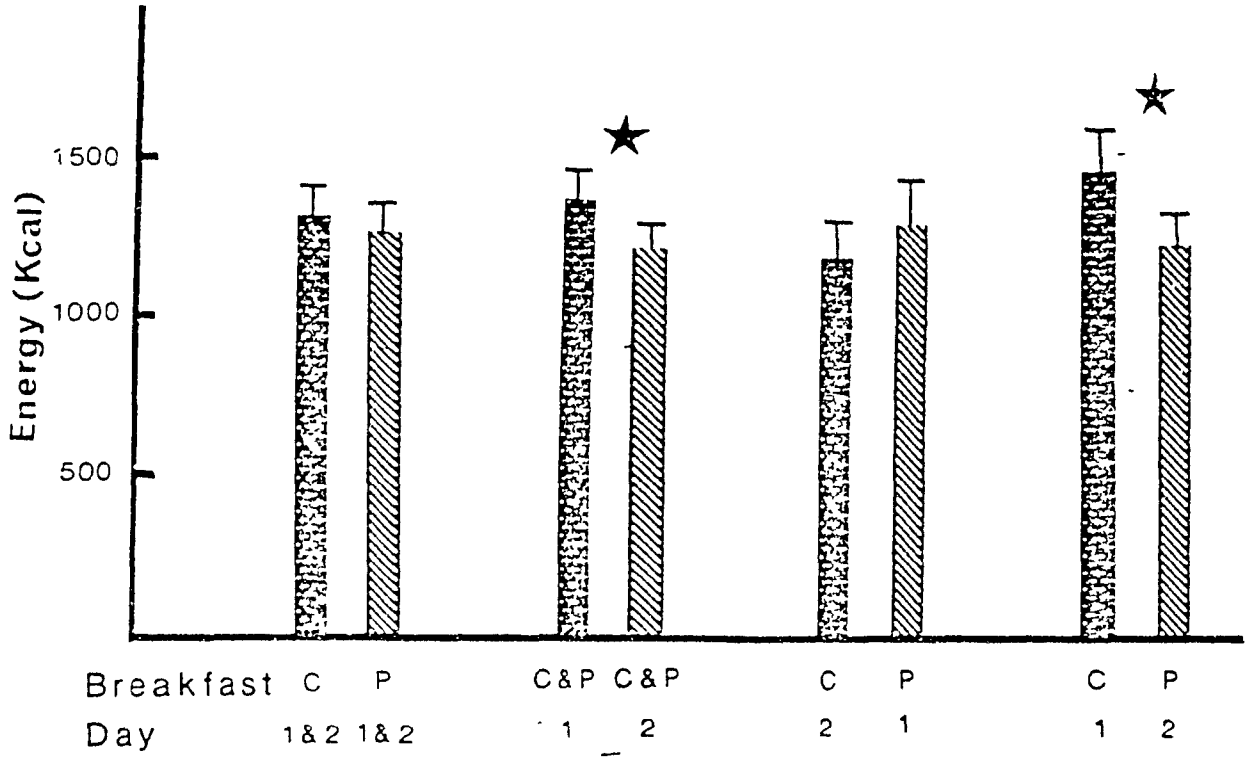

Fig. 1 The effect of carbohydrate or protein breakfasts on energy selection at lunchtime. The key refers to carbohydrate (C) or protein (P) breakfasts given on either day 1 or 2 or both. Thus, the C, 1 & 2 under the first bar refers to values for energy selection at lunchtime in subjects who received a carbohydrate breakfast on either day 1 or 2. Under the third bar C & P, 1 refers to values all subjects on day 1 whether they received a protein or carbohydrate breakfast. Under the fifth bar C, 2 refers to the subjects who received a carbohydrate breakfast on day 2. Values are mean $\pm$ SE. For the first 4 bars i.e. the whole group, n=32. Of these 17 received protein first and 15 received carbohydrate first. A two-tailed paired t-test revealed that, taking the whole group together, subject ate significantly less on the second day ($t=2.39$, $p<0.05$). In the subjects who had the carbohydrate breakfast on the first day, and the protein breakfast on the second day, energy intake at lunchtime was significantly smaller on the second day ($t=2.19$, $p<0.05$).

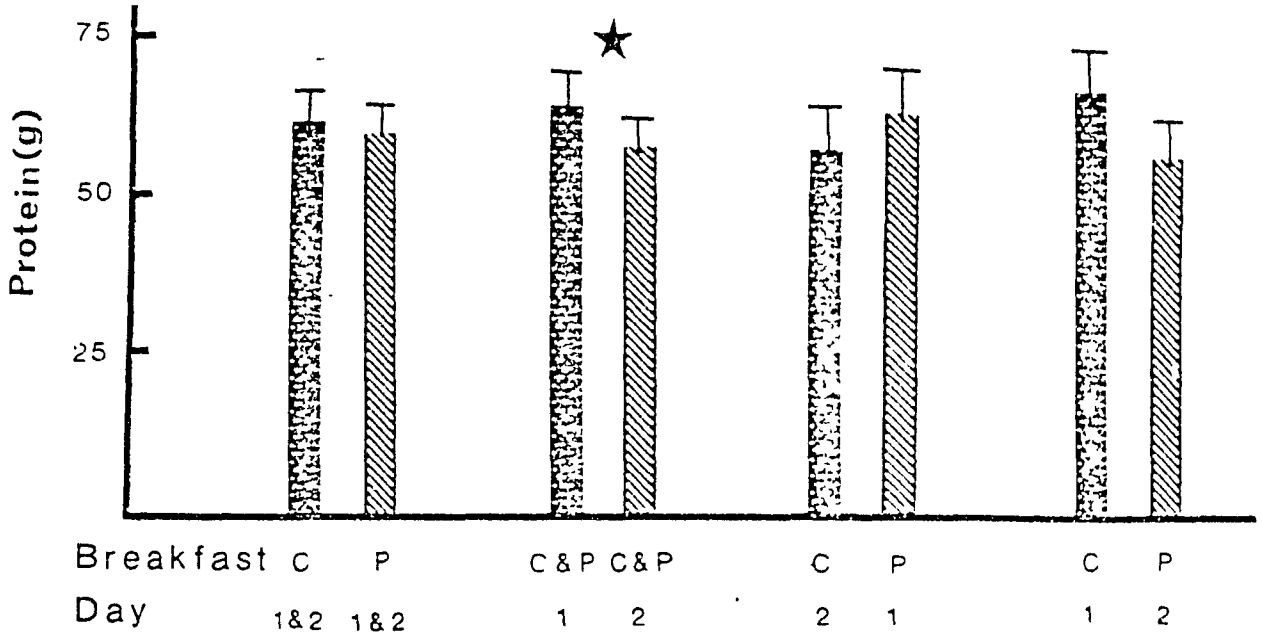

Fig. 2 The effect of carbohydrate or protein breakfasts on protein selection at lunchtime. The explanation of the figure is the same as in the caption to Fig. 1. Taking all the subjects together, significantly less protein was ingested on the second day ($t=2.28$, $p<0.05$). In the subjects who had the carbohydrate breakfast on the first day, and the protein breakfast on the second day, the decline in protein intake on the second day was close to significance ($t=2.0$, whereas a t of 2.4 is necessary to achieve significance).

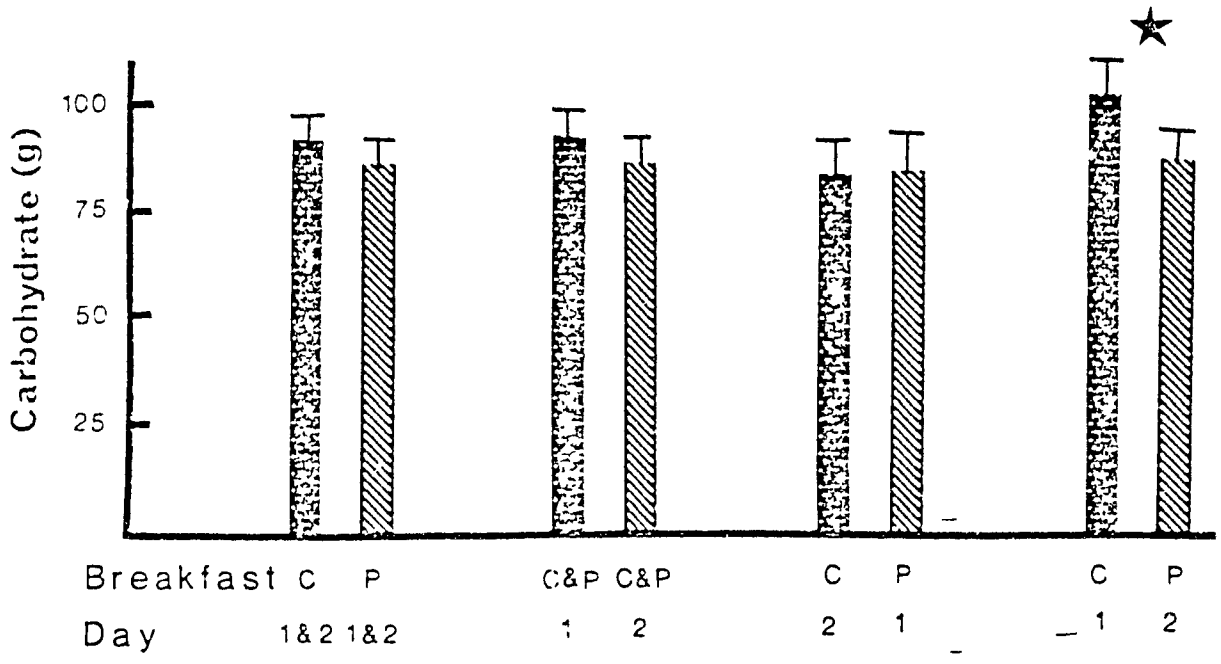

Fig. 3 The effect of carbohydrate or protein breakfasts on carbohydrate selection at lunchtime. The explanation of the figure is the same as in the caption to Fig. 1. In the subjects who had the carbohydrate breakfast on the first day, and the protein breakfast on the second day, carbohydrate intake at lunchtime was significantly smaller on the second day ($t=2.29$, $p<0.05$).

PREFACE TO CHAPTER SEVEN

The experiments in chapter six demonstrated that the ingestion of physiological amounts of protein or carbohydrate did not alter macronutrient selection at the subsequent meal. From these results, one could conclude that protein and carbohydrate did not alter 5HT function. It was suggested that changes in the plasma tryptophan ratio were not of great enough magnitude to alter central 5HT metabolism. To determine if central 5HT could be altered by dietary intake, a more direct measurement of CNS 5HT would be required. In this experiment, amine precursors and metabolites in human lumbar cerebrospinal fluid were measured after the administration of protein or carbohydrate breakfasts. Thus, an attempt was made to answer the question of whether dietary intake can alter CNS 5HT metabolism in humans.

Chapter 7

ACUTE EFFECT OF PROTEIN OR CARBOHYDRATE BREAKFASTS ON HUMAN CEREBROSPINAL FLUID MONOAMINE PRECURSOR AND METABOLITE LEVELS

ABSTRACT

Patients with normal pressure hydrocephalus who had three lumbar punctures during one week ingested either water, a protein breakfast or a carbohydrate breakfast 2.5 h before each of the lumbar punctures. The CSF was analyzed for biogenic amine precursors and metabolites. The protein meal raised CSF tyrosine, a finding consistent with animal data, but did not alter tryptophan or any of the biogenic amine metabolites. The carbohydrate meal increased CSF 3-methyoxy-4-hydroxyphenylethylene glycol, an unexplained finding. The carbohydrate meal did not influence CSF tryptophan, tyrosine, 5-hydroxyindoleacetic acid or homovanillic acid. Our results support the idea that in humans protein or carbohydrate meals do not alter plasma amino acid levels sufficiently to cause appreciable changes in CNS tryptophan levels or 5-hydroxytryptamine synthesis.

INTRODUCTION

Tryptophan, phenylalanine, tyrosine and histidine are the dietary precursors of the neurotransmitters dopamine, noradrenaline, 5-hydroxytryptamine (5-HT) and histamine. For all these precursors variations in their brain levels can, in some circumstances, influence the rate of synthesis of the product neurotransmitters. In the case of 5-HT, the rate-limiting enzyme in the synthetic pathway is tryptophan hydroxylase which is unsaturated at physiological concentrations of substrate. Therefore, administration of tryptophan will increase brain 5-HT in rats (Ashcroft et al., 1965) and 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) of humans (Eccleston et al., 1970). The relationship between dietary tryptophan and brain 5-HT is not as straightforward, due to the nature of the transport system for amino acids across the blood brain barrier. The large neutral amino acids, including tryptophan, compete for the same carrier (Olderdorf and Szabo, 1976). Therefore to estimate tryptophan availability to the brain, the plasma ratio of tryptophan to the sum of it's competitors must be determined (Fernstrom and Wurtman, 1972; Ashley and Anderson, 1975; Fernstrom and Faller, 1978).

In rats, protein and carbohydrate have opposite effects on the plasma tryptophan ratio and on brain tryptophan. Carbohydrate ingestion elicits the secretion of insulin, which causes uptake of the branched chain amino acids into muscle. This lowers the concentration of the competitors in plasma and allows more tryptophan to enter the brain. This may result in increased brain 5-HT (Fernstrom and Wurtman, 1971) although occasionally no effect on brain tryptophan and 5-HT has been reported after carbohydrate (Glaeser et al., 1983). Protein, which

contains high concentrations of other amino acids relative to tryptophan, has been found to lower brain tryptophan (Glaeser et al., 1983) or have no effect (Fernstrom and Faller, 1978). Similarly, a lack of effect (Peters and Harper, 1987) or a decrease (Teff and Young, 1988) has been shown for brain 5HT. In general, meals containing both carbohydrate and protein will tend to lower the plasma tryptophan ratio (Yokogoshi and Wurtman, 1986) and decrease brain tryptophan. However, in one study brain 5-HT was not altered after ingestion of a balanced diet (Perez-Cruet et al., 1972). The reason for the discrepancies between the different studies is not known.

For obvious reasons, the bulk of the experimental work on diet and brain 5-HT metabolism has been performed on experimental animals. In humans, indirect methods must be used. Measurement of the plasma amino acid ratio after dietary manipulation has been employed as an indicator of potential changes in brain tryptophan and 5-HT metabolism (Fernstrom et al., 1979; Ashley et al., 1982; Ashley et al., 1985; Lieberman et al., 1986). These studies all found that protein-containing meals lowered the plasma tryptophan ratio or that carbohydrate meals raised the ratio. Perez-Cruet et al. (1974) found no change in the plasma tryptophan ratio after a balanced meal when they used the total plasma tryptophan level in the calculation. However, they found a decline in the ratio using the free (non-albumin bound) plasma tryptophan ratio. Thus, there is greater unanimity in the data on the changes in human plasma tryptophan ratios after meals than there is in the results on the effects of meals on rat brain tryptophan or 5-HT. However, the significance of the human plasma tryptophan ratio changes is a matter of dispute. Ashley et al. (1985) have argued that any change in the plasma tryptophan ratio will cause a

smaller change in brain tryptophan and a still smaller change in brain 5-HT. They suggest that the magnitude of the changes in human plasma tryptophan ratios after protein or carbohydrate meals would lead to negligible changes in brain 5-HT.

There is only one report examining the effect of diet on tryptophan and 5-HIAA in human cerebrospinal fluid (CSF) (Perez-Cruet et al., 1974). CSF was taken before and four hours after neurological patients ate one of two nutritionally balanced lunches. CSF tryptophan and 5-HIAA were significantly lower in the postprandial samples than in the first CSF samples. In patients who did not eat lunch there was no change in the CSF indoles. The purpose of the present study was to investigate further the effect of meals on human CNS biogenic amine metabolism. We measured amine precursors and metabolites in the CSF of patients who ingested a carbohydrate breakfast, a protein breakfast, or no breakfast.

METHODS

CSF was obtained from patients with normal pressure hydrocephalus (12 men, 3 women, age (mean $\pm$ SD) 70.6$\pm$8.7) undergoing a series of three lumbar punctures. This procedure has been found to improve their neuropsychological performance (Botez et al., 1974) and was the sole reason for hospital admission. The diagnosis of normal pressure hydrocephalus was made on the basis of a CSF scan and a radioisotope cisternogram. Clinical testing revealed mild neuropsychological deficits but no evidence of Alzheimer's disease. Our only alteration of the previously existing protocol was to administer specific dietary treatments. Subjects underwent the three lumbar punctures within a one week period, but with a period of at least one day between each puncture. These were performed at approximately 9:30 a.m.. Between 6:30 and 7:00

a.m., after an overnight fast, subjects were given one of three treatments a) 500 ml of water b) 500 ml of orange juice with sucrose added to give a carbohydrate content of 100g c) 45g of protein in the form of a chocolate pudding. The protein pudding consisted of three 9 oz. packets (with 210 mls of water added) of a protein supplement manufactured by Bariatrix International of Montreal. Each pudding contained 45g of protein, 12g of carbohydrate and 3g of fat. The carbohydrate meal contained more Kcal than the protein meal because humans normally take in more carbohydrate than protein. The treatments were given in a counterbalanced order. Therefore for each subject, a CSF sample was obtained after fasting and after protein and carbohydrate ingestion. For a few patients, we were not able to administer all three treatments. These subjects were only included if a CSF sample was obtained after fasting. The number of subjects included in the carbohydrate group was 14, while in the protein group there were 1[illegible]. CSF was withdrawn from a needle inserted between vertebrae L3 and L4 with the patient in the sitting position and was allowed to drip from the needle directly into the tube. The first 4ml were taken for routine analysis, while the next 5ml were collected as a single fraction for research purposes. For some of the patients (for carbohydrate, n=5; for protein, n=7) a blood sample was taken immediately before CSF removal and collected into heparinized tubes for analysis of plasma amino acids. Both CSF and blood samples were taken to the laboratory on ice, and frozen at -70°C. The protocol was approved by the Ethics Committees of Hotel Dieu Hospital and the Department of Psychiatry, McGill University.

The free plasma tryptophan concentration was taken as the concentration of tryptophan in an ultrafiltrate of plasma prepared at

25°C under 5% CO_2 in an Amicon MPS-1 centrifugal ultrafilter using YMT membranes. Ultrafiltration through YMT membranes removes protein from the plasma. Thus, tryptophan bound to albumin will be removed and only the free (i.e. non-albumin bound) tryptophan remains in the ultrafiltrate. Tryptophan in the ultrafiltrate and in the plasma were measured by the fluorometric method of Denckla and Dewey (1967). Plasma was prepared for amino acid analysis by adding 50mg of 5-sulfosalicylic acid to 1ml of plasma. Samples were vortexed, left for 1 h at -4°C and centrifuged at 12,000g for 10 min. The supernatant was mixed with 0.3N Lithium Hydroxide in a ratio of 2.5:1.0, and assayed on an LKB Alpha Plus amino acid analyzer with high resolution column.

Tryptophan, tyrosine, 5HIAA and homovanillic acid (HVA) in the CSF were measured using the method of Anderson et al. (1979). This method involves direct injection of the CSF into a high performance liquid chromatograph, separation of the compounds on a reverse-phase column (30cm X 3.9 mm of 10 u micro-Bondapak C18 from Water Associates Inc.) and fluorometric and electrochemical detection. 3-Methoxy-4hydroxyphenylethylene glycol (MHPG) was measured by reverse-phase HPLC with electrochemical detection (Anderson et al., 1981). The effect of protein and carbohydrate breadfasts on plasma amino acids and CSF indoles were analyzed using a paired t-test. Values after protein or carbohydrate pretreatment were compared with the fasted control values of the same subjects.

RESULTS

In Table 1, the effects of protein and carbohydrate meals on plasma amino acid neurotransmitter precursors are shown. The carbohydrate meals lowered all the amino acids except for total tryptophan, but the effect

was statistically significant only for phenylalanine. In contrast, protein significantly increased all the amino acids except histidine. The effect of the dietary treatments on the ratios of the neutral amino acids to the sum of the competitors is shown in Table 2. In these ratios the sum of the competitors included tryptophan, tyrosine, phenylalanine, valine, leucine, isoleucine, histidine and methionine with the amino acid in question being excluded from the group. The carbohydrate breakfast raised the ratio of total tryptophan, tyrosine and histidine by 34% and 33% respectively, but the result was statistically significant only for the histidine ratio. Protein resulted in a significant lowering of the tryptophan, free tryptophan and histidine ratios. Tyrosine and phenylalanine were not changed significantly.

Fig. 1 illustrates the effect of carbohydrate on the biogenic amine precursors and metabolites in CSF from 14 subjects. Carbohydrate caused a significant increase in MHPG levels but did not influence tryptophan, tyrosine, 5-HIAA or HVA. The effect of protein on CSF measures in 15 patients is shown in Fig. 2. Protein increased CSF tyrosine levels significantly but did not affect the other compounds. After the control treatment there was a significant correlation between CSF 5HIAA and HVA ($r=0.72$, $p<0.01$) and this remained after the carbohydrate ($r=0.88$, $p<0.01$) and protein ($r=0.93$, $p<0.01$) meals.

DISCUSSION

Studies on experimental animals have established that there is a relationship between tryptophan in brain and CSF (Young et al., 1976), while human autopsy material has been used to demonstrate a relationship between 5HIAA and HVA in brain and CSF (Stanley et al., 1985). However,

a variety of clinical factors can influence CSF amine metabolite levels, and such measurements are a rough index rather than a direct measure of the turnover of the parent amines in the CNS. Nonetheless, changes in amine synthesis due to alterations in precursor availability have been detected through CSF measurements. Increases in CSF 5-HIAA after tryptophan loading have been seen in several studies (e.g. Eccleston et al., 1970; Young and Gauthier, 1981), and an increase in CSF HVA has been detected in patients with Parkinson's disease after tyrosine administration (Growdon et al., 1982). Thus, in the present study measurements on CSF should have been able to detect any important changes in CNS biogenic amine metabolism due to diet-induced alterations in precursor availability. Factors such as age, sex and height of the patient, volume of CSF removed and movements of the patient which can influence amine metabolite levels in CSF would not have been important for the data reported in this paper because each patient acted as his or her own control, the volume of CSF taken was constant and the patients' movements were limited.

One disadvantage of CSF studies is the lack of neuroanatomical resolution of the results. Metabolite levels in lumbar CSF reflect metabolism in both the spinal cord and various parts of the brain. The contribution from the spinal cord differs for the different metabolites (Garelis et al., 1974). However, alterations in precursor availability will not necessarily produce alterations in amine synthesis that are limited to specific regions. Thus, tryptophan administration increases 5-HT levels in all areas of rat CNS (Moir and Eccleston, 1968). Any diet-induced alteration in amine metabolism would also likely occur throughout the CNS if it was mediated by an alteration in precursor

availability.

Our results show that protein or carbohydrate meals failed to influence CSF tryptophan or 5HIAA. Thus, our data support the contention of Ashley et al. (1985) that, in humans, meals do not alter plasma tryptophan enough to cause important changes in CNS 5-HT metabolism. They suggest that a rise of 50% in the plasma tryptophan ratio is needed to produce significant increases in 5-HIAA. In our study the carbohydrate meal produced a 47% increase in the plasma tryptophan ratio. Because of the small number of patients from whom it was possible to get these plasma measurements this change in the ratio was not statistically significant. The significant decline of 25% in the plasma tryptophan ratio after the meal is smaller than the 30% fall in the ratio which Ashley et al. (1985) estimate is needed to cause a decline in 5-HIAA.

Our results do not, at first sight, agree with the results of Perez-Cruet et al. (1974). However, one important difference between our results and those of Perez-Cruet et al. (1974) is the length of time the patients fasted before the meal or lumbar puncture. In their study patients who remained fasting before the second lumbar puncture had not eaten for nearly 24 hours, almost twice the length of fast in our study. Some aspects of the results of Perez-Cruet et al. (1974) are puzzling. First, they did not find a decline in the plasma tryptophan ratio using the value for total plasma tryptophan, after the subject ate a protein-containing meal. Although they did find a significant decline in the ratio when they used the free (non-albumin bound) plasma tryptophan value, the lack of change in the total tryptophan ratio contradicts a variety of other studies (Fernstrom et al., 1979; Ashley et al., 1982; Ashley et al., 1985; Lieberman et al., 1986), as well as this study.

Furthermore, the effect of albumin binding on uptake of tryptophan into the brain is probably small (Etienne et al., 1976; Yuwiler et al., 1977). Second, the changes in CSF 5-HIAA are much larger than would be expected with the magnitude of changes in CSF tryptophan seen in the study of Perez-Cruet et al. (1974). In humans as in rats tryptophan hydroxylase is normally about half saturated with tryptophan (Young and Gauthier, 1981). Under these circumstances a 20% decline in tryptophan levels would lead to a 11% decrease in the rate of 5-HT synthsis. However, in two experiments Perez-Cruet et al. (1974) found that meal-induced declines in CSF tryptophan of 20% and 21% led to decreases in CSF 5-HIAA of 20% and 35%. It is unlikely that the entire decline in CSF 5-HIAA in these two experiments was due to a decline in tryptophan availability. In spite of the factors discussed above the discrepancy between the results of our study and those of Perez-Cruet et al. (1974) remain somewhat puzzling. Nonetheless we feel that in view of the agreement between our data and the conclusions of Ashley et al. (1985), we can safely conclude that, in the absence of prolonged fasting as in the study of Perez-Cruet et al. (1974), protein or carbohydrate meals will not alter human CNS tryptophan levels enough to have any important effect on 5-HT.

In spite of the absence of any change in CSF tryptophan, the protein meal increased CSF tyrosine. This is consistent with animal data which showed that the increase in brain tyrosine after a 40% protein meal was 3.3 fold, a magnitude of change much greater than that which occurs with tryptophan (Glaeser et al., 1983). However, the increase in tyrosine did not lead to any increase in catecholamine synthesis. This is what would be expected as tyrosine hydroxylase is closer to saturation with tyrosine

than tryptophan hydroxylase is with tryptophan. In addition tyrosine hydroxylase is normally controlled by feedback inhibition and tyrosine availability usually only becomes an important factor in the control of catecholamine synthesis when catecholamine neurons are distributed from their normal equilibrium (Wurtman et al., 1981).

The significant changes in the histidine ratio after protein or carbohydrate meals are qualitatively different from those seen in rat brain, presumably reflecting differences in the control of histidine metabolism between rats and humans. In this study the plasma histidine ratio increased with the carbohydrate meal and declined with the protein meal, while in the rat carbohydrate decreased, and a 40% protein meal increased brain histidine (Glaeser et al., 1983). The magnitude of the decline in the histidine plasma ratio after protein (56%) may well have been sufficient to alter brain histamine synthesis, as histamine metabolism is sensitive to changes in presursor availability (Imura et al., 1986).

The reason for the increase in MHPG after carbohydrate is unknown, but it does not seem to be due to altered tyrosine availability. As 10 comparisions were made on CSF measures (five metabolites each with two different meals) there is a reasonable probability of finding one false positive at the 0.05 level. If the effect is a real one it may be mediated by the vagus nerve. In the rat subdiaphramatic vagotomy can alter brain catecholamine metabolism and the vagus nerve has receptors for many different dietary consistuents. Direct effects of nutrients on brain cells is also a possibility as there are glucose-sensitive neurons in the hypothalamus (Shimizu et al., 1983). Glucose has been reported to suppress firing of central dopaminergic neurons (Saller and Chiodo,

1980), but this finding has not been replicated (Trulson et al., 1983). In this study we found no effect of a carbohydrate meal on CSF HVA.

Our results have implications for the design of studies which involve measurement of amine metabolite levels in human CSF. As diet-induced changes in brain amine metabolism are likely to be negligible, precautions concerning diet need not be too stringent. Controlling the diet of patients in the days before a lumbar puncture is unlikely to be necessary. The differences between our results and those of Perez-Cruet et al. (1974) suggest that a 24 hour fast, as in the study of Perez-Cruet et al. (1974), may bring about unphysiological changes in CNS metabolism. Probably the only necessary precaution is to study the patients after an overnight fast, although it may well be that, in many situations, even an overnight fast is unnecessary.

The important conclusion of our study is that protein and carbohydrate meals did not alter CNS tryptophan or 5-HT metabolism. Although macronutrient ingestion can alter 5HT in rodents, such changes are unlikely to occur in humans. This species difference exists presumably because of differences in amino acid metabolism, which cause the alterations of the plasma tryptophan ratio after a meal to be smaller in humans than in rats. This illustrates the problems inherent in extrapolating from one species to another. Indeed it is important not to generalize too widely from the results of the present study. The patients studied had a mean age of 71, suffered from normal pressure hydrocephalus and had mild neuropsychological deficits. However, the fact that there was a significant correlation between CSF 5HIAA and HVA, a finding in most human CSF studies (Agren et al., 1986), suggests that their brain amine metabolism was relatively normal. Nonetheless, our

finding does not mean that food would not influence brain amine metabolism in younger normal subjects ingesting larger meals. They do suggest that in many circumstances food is unlikely to influence brain amine metabolism in humans, and that if changes do occur in any circumstances they are likely to be small. This means that the effects of mood on CNS function are unlikely to be mediated by alterations in 5-HT function which occur as a result of altered brain tryptophan. Spring et al. (1983) looked at the effects of carbohydrate or protein meals in humans. They found that the carbohydrate meals tended to have a sedative effect relative to the protein meals and attributed this effect to a carbohydrate-induced increase in 5-HT function. If our conclusions are correct another mechanism must be invoked to explain this type of food-mediated alteration in brain function.

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Table 1. Effect of carbohydrate and protein breakfasts on plasma amino acids

Treatment	Plasma concentration (nmol/ml)				
	Total Tryptophan	Free Tryptophan	Tyrosine	Phenylalanine	Histidine
Control	54.3±13.2	7.56±1.90	58.9±19.8	47.4±2.7	65.3±20.4
Carbohydrate	56.1±27.4	6.46±1.00	36.4± 9.3	32.3±5.1**	59.4±13.7
Control	54.8±10.8	8.25±1.30	56.8±17.0	48.8±5.0	63.7±16.9
Protein	82.8±19.6**	11.6 ±1.9*	123 ±23***	81.1±16.2*	58.3±34.5

Values shown are mean ± SD, n=5 for carbohydrate group, n=7 for protein group. Free tryptophan refers to the non-albumin bound level, while total tryptophan refers to the free plus bound levels.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus relevant control group.

Table 2. Effect of carbohydrate and protein breakfasts on plasma amino acid ratios

Treatment	Total Trp/NAA	Free Trp/NAA	Tyr/NAA	Phe/NAA	His/NAA
Control	0.093±0.019	0.012±0.004	0.101±0.043	0.081±0.010	0.110±0.040
Carbohydrate	0.137±0.066	0.014±0.002	0.115±0.071	0.076±0.018	0.146±0.026**
Control	0.093±0.017	0.013±0.002	0.096±0.036	0.082±0.008	0.107±0.034
Protein	0.070±0.018**	0.009±0.001*	0.106±0.012	0.071±0.019	0.047±0.025***

Values shown are mean ± SD, n=5, for carbohydrate group, n=7 for protein group.
NAA (large neutral amino acids) = sum of Trp, Phe, Tyr, His, Val, Leu, Ileu, Met (omitting amino acid in the numerator of each ratio)
* P<0.05, ** P<0.01, *** P<0.001 versus relevant control group.

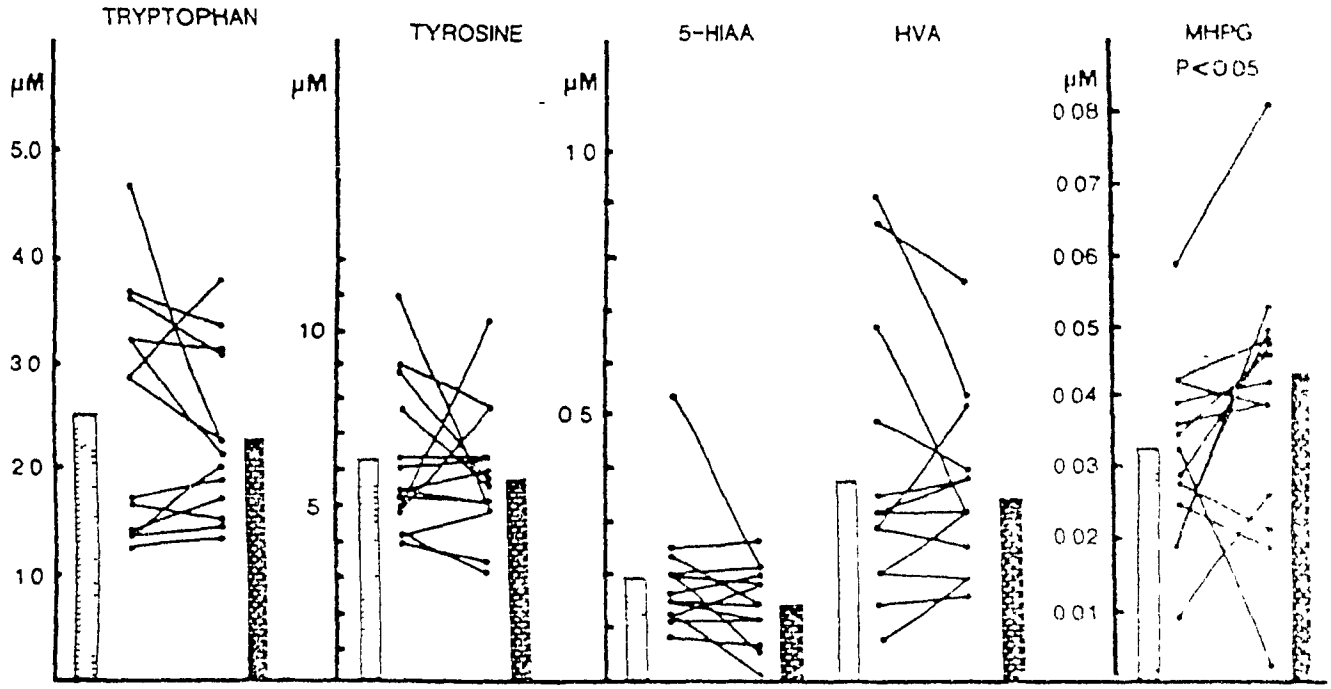

Fig. 1. The effect of a carbohydrate breakfast on amine precursors and metabolites in CSF. The points connected by a line give data for a single patient. In each case the value at the left end of the line is the value after ingestion of water (control) and the value at the right end of the line is the value after ingestion of a carbohydrate breakfast. There was a significant ($P<0.05$) increase in CSF MHPG after ingestion of the carbohydrate meal. All other changes were not significant.

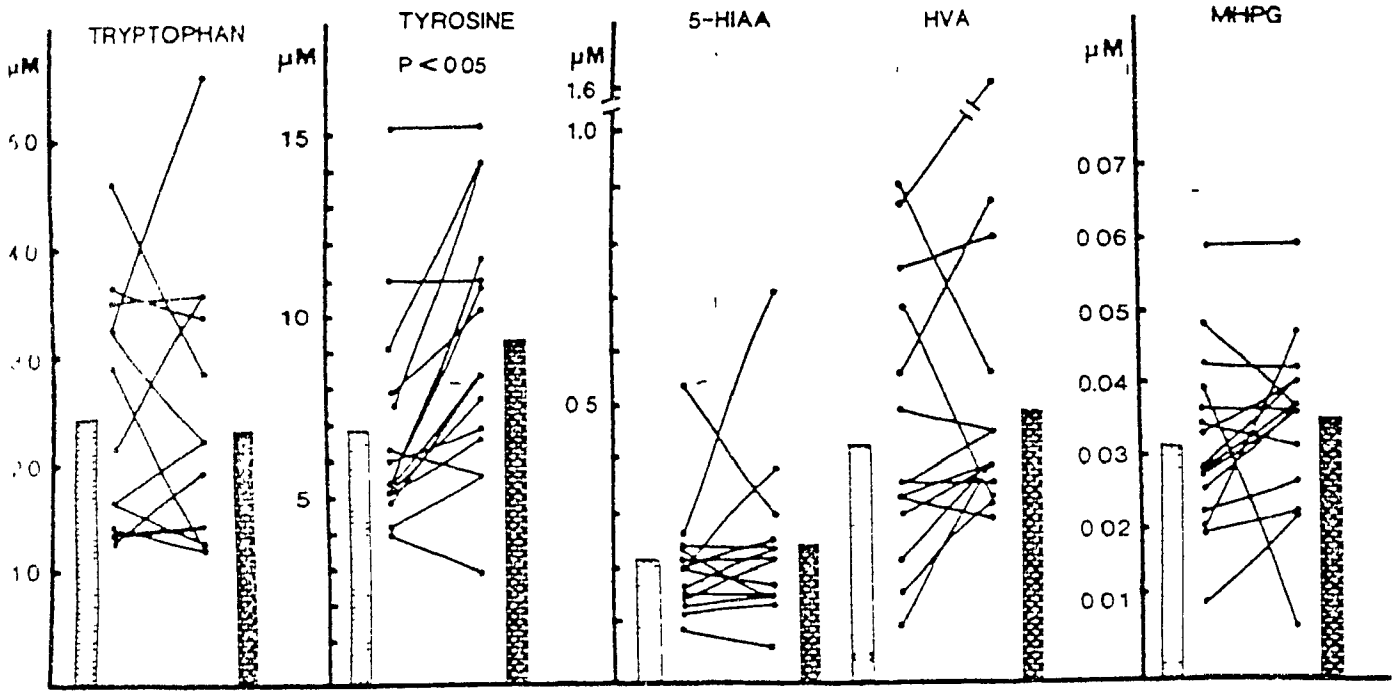

Fig. 2. The effect of a protein breakfast on amine precursors and metabolites in CSF. The points connected by a line give data for a single patient. In each cause the value at the left end of the line is the value after ingestion of water (control) and the value at the right end of the line is the value after ingestion of a protein breakfast. There was a significant ($p<0.05$) increase in CSF tyrosine after ingestion of the protein meal. All other changes were not significant.

Chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

The original premise of the work was to determine the extent of dietary influences on 5HT metabolism and ultimately, function. The assumption, derived from the literature, that pure protein and carbohydrate would affect 5HT metabolism in opposite ways, through alterations in precursor availability, was upheld by the results of the first animal experiment. Both brain and pancreatic tissue 5HT were found to be responsive to macronutrient administration. Determining the functional significance of these changes was the next logical step. Though the possibility of pancreatic 5HT modulating insulin release was a concept worth exploring, our area of expertise was in neurochemistry and therefore we decided to approach the problem of evaluating functional 5HT in the brain. The measurement of 5HT in the cisternal CSF of the rat was found to be an appropriate index of functional 5HT. Initially, we looked at whether functional changes would parallel the metabolic alterations observed after treatment with dietary components. Tryptophan administration, under normal environmental conditions, did not increase CSF 5HT, suggesting that large increases in precursor availability were not sufficient to alter 5HT release appreciably. An increase in functional 5HT could be observed after tryptophan, if the animals were placed in a novel environment in a darkened room. This was used as a behavioral arousal paradigm to optimize the chances of seeing an effect of the macronutrients on 5HT release. Despite, the optimal conditions and the repeated administration of pure protein and carbohydrate, CSF 5HT was not significantly altered. Therefore, it was concluded that in rats, the metabolic changes resulting from macronutrient ingestion were not of appreciable functional significance.

In humans, similar questions were asked. Results revealed some similarities and some differences from the animal work. The important similarity was that protein and carbohydrate do not influence brain 5HT function due to changes in precursor availability. However, in humans, unlike in rats, this is because protein and carbohydrate fail to influence brain 5HT metabolism to any extent.

As direct measurement of functional 5HT in humans was not possible, macronutrient selection, a behavior demonstrated to be mediated by 5HT in animals, was monitored as a means of assessing functional changes in humans. The first human experiment supported the original hypothesis that changes in precursor availability could alter 5HT function. Thus, depletion of peripheral tryptophan by the administration of a tryptophan-deficient mixture of amino acids resulted in less protein selected at the following meal. An effect on macronutrient selection was not observed however, when normal dietary constituents were ingested, suggesting that in humans, 5HT is not involved in the regulation of macronutrients on a meal to meal basis. Furthermore, plasma tryptophan ratios considered to be an index of tryptophan availability to the brain, were only significantly increased after the ingestion of pure carbohydrate. Small amounts of protein were found to inhibit the rise in the ratio normally observed after carbohydrate ingestion. As virtually all foods contain more than 4% protein, differential effects on the ratio after food intake are unlikely. In addition, extrapolating from animal data, the rise in the ratio was not thought to be of sufficient magnitude to alter CNS 5HT metabolism. This was later verified by the final experiment on humans in which amine precursors and metabolites were measured in human lumbar CSF after protein and carbohydrate ingestion.

CNS tryptophan and 5HT were uneffected by dietary intake. Therefore, it was concluded that though 5HT may be a modulator of macronutrient selection, in humans, this is not a mechanism under dietary control, as ingestion of protein or carbohydrate do not alter central 5HT metabolism or function.

The results of both the animal and human work led to the same final conclusion; ingestion of the macronutrients, protein and carbohydrate on a physiological scale do not alter central 5HT function. This certainly was not our original hypothesis but one, with the advantage of hindsight, which is not difficult to accept. An objective review of the literature, in conjunction with the data presented in the thesis, leds one to surmise that in some ways, the concept of 5HT being involved in a feedback loop has been propagated by it's intellectual appeal rather than by objective assessment of the evidence. Though dietary intake can alter 5HT metabolism in rats (Fernstrom and Wurtman, 1971; Fernstrom and Faller, 1978; Teff and Young, chapter 2), this is not a robust phenomenon. Published reports of a lack of effect on brain tryptophan and 5HT are evident (Glaeser et al., 1983; Peters and Harper, 1987).

While pharmacological manipulations support the idea of a feedback loop existing in animals, i.e. increases or decreases in brain 5HT resulting in a compensatory selection of either protein or carbohydrate (Ashley et al., 1979; Li and Anderson, 1984; Wurtman and Wurtman, 1979), acute dietary experiments examining food selection without the administration of either tryptophan or monoamine oxidase inhibitors are very few. In addition, the demonstration that as little as 5% protein can inhibit the rise in the plasma tryptophan ratio (Yokogoshi and Wurtman, 1986), which has also been shown in humans (chapter 6) suggests

that neurochemically, only pure carbohydrate foods can be differentiated from protein. The majority of the self-selection experiments offered animals a choice of two diets, usually containing 10 and 60% protein, which based on the above experiment, would not have contrasting effects on brain 5HT.

Most of the studies looking at food selection in animals are chronic dietary experiments in which animals have been maintained on one specific diet or allowed to select from two diets for an extended period of time (Ashley and Anderson, 1975; Anderson and Ashley, 1977). Even within the chronic dietary work, there are a number of reports suggesting that 5HT is not involved in the regulation of macronutrient intake (Peters and Harper, 1984; Fernstrom et al., 1985; Leathwood and Ashley, 1983). At this point, several issues can be raised: 1) the pharmacological experiments verify the role of 5HT in macronutrient selection, but do not support the hypothesis that changes in precursor availability brought about by dietary intake, can alter functional 5HT which in turn can effect food selection 2) interpretation of chronic dietary experiments is difficult since one does not know whether selection is a result of altered brain levels or if brain levels are a result of food selection. 3) The difference between chronic and acute dietary experiments should be emphasized. Induction of peripheral enzymes after protein intake has been demonstrated (Anderson et al., 1968) and could play an important role in limiting precursor availability. Thus, the evidence for protein and carbohydrate altering 5HT levels in the brain, which in turn alters food selection in animals, is not that strong.

The situation in the human literature is similar, but the misinterpretation is derived from a different source. Despite evidence

and discussion suggesting that, in man, changes in the plasma tryptophan ratio after ingestion of dietary components were unlikely to alter central 5HT metabolism (Ashley et al., 1982; Ashley et al., 1985; Curzon and Sarna, 1984), many of the behavioral effects of food have been attributed to changes in brain 5HT metabolism, mediated by precursor availability (Lieberman et al., 1986; Spring et al., 1983). If dietary intake does not alter the plasma tryptophan ratio sufficiently to effect central 5HT metabolism, as we have demonstrated (Chapter 6) and others have suggested (Ashley et al, 1985; Curzon and Sarna, 1984), then it is unlikely that the various cognitive and psychological effects observed after dietary intake are mediated by brain 5HT. Numerous other biochemical compounds are candidates as mediators of the behavioral effects of foods. Firstly, there are the other amino acid precursors, tyrosine, phenylalanine and histidine which have the capability of altering the neurotransmitters, dopamine, noradrenaline and histamine. However, with the possible exception of histidine, which has not been studied in humans, it is doubtful that the effects are mediated by tyrosine or phenylalanine since they are less susceptible to dietary intake than tryptophan. Another possibility is that glucose, which has been shown to have a direct effect on dopamine neurons in the substantia nigra (Saller and Chiodo, 1980) and an indirect effect on the vagal nerve (Shimizu et al., 1983). In fact, it has been demonstrated that the vagal nerve can be stimulated by a variety of gut hormones thought to be involved in satiety (Morley et al., 1984; Smith and Gibbs, 1984) and also by amino acids (Li and Anderson, 1984). Many of the intestinal peptides such as neuropeptide Y, bombesin, cholecystokinin and somatostatin have been suggested as regulators of food intake and probably exert direct

effects on the brain. If they influence food intake via systems in the brain, then the possibility exists that they influence other aspects of brain function.

Though no effect of dietary intake on central 5HT metabolism and function was observed under normal circumstances in normal people, this does not rule out the possibility that in some populations or in some situations, an effect could be demonstrated. People with certain psychiatric disorders may be more susceptible to macronutrient intake, for example, patients displaying a trend to impulsivity, a condition associated with lowered 5HT function. Also, environmental conditions could perhaps enhance the effect of diet, as illustrated by the behavioral arousal paradigm used to enhance the effect of tryptophan administration in rats (chapter four). Therefore, though precursor availability was not found to alter central 5HT function, this conclusion is applicable only within the context of the specific experiment.

Some of the problems associated with the measurement and determination of 5HT function in animals and humans have been discussed throughout the thesis. These difficulties point to areas which require exploration and act as suggestions for the direction of future work. One subject repeatedly mentioned, which is of crucial importance, is experimental design. Especially in humans, psychological factors can exert significant effects on food intake and may override the subtle physiological mechanisms. A prime example of this is the suppression of food intake on the second trial of the dietary selection study (Chapter six). Eagerness for payment combined with the lack of novelty of the foods offered, caused a reduction in food intake that was greater than any physiological effect on macronutrient ingestion. Psychological as

well as sensory factors are often ignored but are essential components in regulating dietary intake. The design of experiments which take into account these elements is obligatory if subtle changes brought about by food ingestion are not to be overlooked.

Individual variability in response is a problem common to both the animal and human experiments. If one could determine the factors responsible for this variability, then a greater understanding of the mechanisms involved in precursor availability would develope. Rat CSF 5HT manifests a large individual variation even wihin the same species, weight, time of day, treatment etc. An attempt was made to evaluate these factors by assessing the effect of stress, starvation and light on CSF 5HT (Teff and Young, unpublished data). In some cases trends were observed, but none of the above parameters significantly influenced CSF 5HT levels. The individual animal's response to stress may be an important determinant of the variation. Thougn restraint stress had no effect on CSF 5HT, it is possible that other kinds of stress would be effective. The acute withdrawal of CSF does not allow for comparisons between baseline and posttreatment values within one animal. Hutson et al.(1985) have monitored the effect of tryptophan administration on CSF tryptophan and 5HIAA over an extended period of time, using a chronically implanted cannula. The individual response to tryptophan administration is very striking. In some animals, huge increases were observed, while in others, the rise was barely above baseline variation. What accounts for these large differences? Many factors may be responsible. Genetic variation in enzyme affinity is one possibility. Tryptophan uptake into the brain is another. The physiological parameters which regulate amino acid uptake have not been explored. There is one small piece of evidence

which suggests that stress may influence uptake of amino acids into the brain. Kennett et al. (1986) found that most of the brain amino acids were elevated after immobilization. Though the mechanism for this is unknown, an alteration in the kinetics of transport at the blood-brain barrier, possibly mediated by hormones, was postulated. It is essential to determine the factors regulating amino acid uptake. Much emphasis has been placed on the plasma tryptophan ratio as an index of tryptophan availability to the brain. If in some circumstances this is not the appropriate measure (as has been suggested in the free vs. bound tryptophan controversy), then much experimental data will have to be reevaluated.

Individual variation is also evident in human responses. In some human subjects, large changes were observed in dietary selection while in others, food intake remained constant. This may be related to either baseline plasma tryptophan or tryptophan ratios. Franklin et al. (manuscript in preparation) found that basal tryptophan levels exhibited a high correlation with post-operative morphine requirements. It is possible, due to various physiological factors that subjects (and perhaps animals) could be divided into responders and non-responders to precursor availability. Experiments designed to test this hypothesis might prove very useful for future work.

The final conclusion of the thesis suggests that in rats, protein and carbohydrate can alter 5HT metabolism but not function, while in humans, neither central 5HT metabolism or function is effected by dietary macronutrients. In some ways, this has been disappointing. It seems that the concept of dietary intake influencing brain metabolism has come full circle from initial disbelief, which required proof of the

unsaturation of tryptophan hydroxylase, leading to a general acceptance of the behavioral effects of food. Unfortunately, this idea has been readily taken up by the lay press and general populace and now, based on sound scientific data, must be qualified. The results presented here should lay to rest some of the myths and beliefs surrounding carbohydrate intake and in that way, are very satisfying. We have only just begun to comprehend the vast complexity of the consequences of food intake and the mechanisms involved in its regulation. Integration of the many different areas of science; including genetics, neurochemistry, nutrition, psychology, and physiology will be necessary before a complete understanding is reached.

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CLAIMS FOR ORIGINAL RESEARCH

Chapter 2. In rats, protein administration was shown to lower brain 5HT levels. Dietary effects were also demonstrated on pancreatic tissue. Acute protein intake lowered pancreatic 5HT and carbohydrate raised it. These effects seem to be mediated by competition between tryptophan and other amino acids for entry from blood to pancreas, suggesting the existence of a "blood-pancreas" barrier. The pineal was shown to be unresponsive to dietary manipulation.

Chapter 3. A method for the direct measurement of 5HT in rat cisternal cerebrospinal fluid was validited as an index of functionally active 5HT by the administration of a variety of drugs known to act on 5HT function. Antidepressant drugs werre shown to increase CSF 5HT three fold, while treatments which cause the serotonin behavioral syndrome increased CSF 5HT 16-20 fold.

Chapter 4. Tryptophan administration did not increase rat CSF under normal circumstances. However, tryptophan was also given to animals which had been aroused by placing them in a novel environment in the dark. Arousal is known to increase firing of 5HT neurons and under these circumstances tryptophan increases CSF 5HT. This suggests that alterations in the brain levels of precursors such as tryptophan will influence release of the product neurotransmitter only when the relevant neurons are firing at a high rate. No effect on functional 5HT, as measured by CSF 5HT, was observed after either protein or carbohydrate were given to aroused animals. The animal experiments indicate that

although dietary intake can alter central 5HT metabolism, the changes are too small to alter 5HT function.

Chapter 5. In humans, the administration of a tryptophan deficient amino acid mixture in the morning, significantly decreased protein selection at lunch time. This demonstrated that 1) large changes in tryptophan availability can alter a behavior thought to be mediated by 5HT 2) 5HT is involved in the modulation of macronutrient selection in humans.

Chapter 6. Protein and carbohydrate meals of similar sensory qualities were developed. This made possible a study of the metabolic effects of meals differing in macronutrients which eliminated any effects that were due to differences in taste sensation or cognitive attribution. The ingestion of a breakfast of either protein or carbohydrate had no effect on overall energy or macronutrient selection at lunchtime. This suggested that functional 5HT was not altered by dietary intake and that, on a physiological level, 5HT was not regulating macronutrient selection on a meal-to-meal basis. The addition of 4% protein to a carbohydrate breakfast was found to inhibit the rise in the plasma tryptophan ratio, indicating that as far as the plasma tryptophan ratio is concerned, only pure carbohydrate foods can be differentiated from protein foods. From the change in the tryptophan ratio after ingestion of breakfasts containing varying amounts of protein, it was postuated that the degree of change exhibited by the tryptophan ratio was not of sufficient magnitude to alter central 5HT metabolism.

Chapter 7. Pure carbohydrate or protein breakfasts were not found to alter CSF tryptophan or 5HIAA in humans, indicating that the dietary macronutrients do not alter central 5HT metabolism in humans.