COMPARTMENTAL DISTRIBUTION OF AMINO ACIDS AND MIDDLE MOLECULAR SUBSTANCES IN NORMAL AND GALACTOSAMINE INDUCED FULMINANT HEPATIC FAILURE RATS

by

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To my parents and my wife.

ABSTRACT

The major part of this thesis is to obtain basic information in regard to changes of amino acids and middle molecular substances in fulminant hepatic failure (FHF) using a galactosamine induced rat model. The changes of these substances after hemoperfusion were also studied.

A generalized elevation of amino acid concentrations is demonstrated in the systemic blood plasma, portal plasma, CSF, cerebrum, liver, kidney and skeletal muscle tissues in GalN-FHF. The skeletal muscle is found to constitute the greatest source of accumulated amino acids in FHF. The other tissues also contribute to the increased amino acids in the body as a whole. The increase in aromatic amino acids (AAA) tyrosine, phenylalanine and free and total tryptophan were the most striking among all the amino acids, including branched chain amino acids (BCAA), in all the tissues studied. The molar ratio of BCAA/AAA was found significantly reduced in all the tissues studied. γ-amino butyric acid was found significantly increased in the cerebrum and the brain stem. Most of the increased tryptophan in plasma, and almost 100% of the increased tryptophan in the brain were in the free form (non-protein bound). The increase in tyrosine concentration in plasma was closely correlated with tyrosine in the brain. Hemoperfusion using collodion coated activated charcoal (CAC) significantly reduced a number of aromatic amino acids in the plasma. This was followed by a significant reduction of the AAA in CSF, but not in the brain, of GalN-FHF rats. Hemoperfusion using tyrosinase immobilized within artificial cells selectively reduced tyrosine in the plasma but did not influence the tyrosine level in the brain. Hemoperfusion procedures resulted in a high plasma clearance for the aromatic amino acids. The results also suggested the loss of the blood brain barrier for amino acids across the capillaries. However, transport mechanisms between brain cells themselves and interstitial fluid seems to be maintained.

The buildup of middle molecular metabolites (MW 500-2,000) was demonstrated as the elevation of middle molecule peak 7g in the plasma and brain extract samples from GalN-FHF rats using serial liquid chromatography. CAC hemoperfusion significantly reduced the levels of 7g fraction in both plasma and brain extract samples. The fraction of subpeak 7g was found to contain peptidic substances. SDS-polyacrylamide gel electrophoresis further revealed the peptide nature of some of the middle molecule substances. The estimated molecular weight of these peptides was in the range of 1,300 and 1,400. Radio-immunoassay study indicated the increase of substance P (MW 1,345) in the plasma of GalN-FHF rats.

RESUMÉ

L'essentiel de cette thèse réside dans l'obtention de données fondamentales sur les changements subis par les acides aminés et les substances de poids moléculaire moyen chez les rats atteints d'insuffisance hépatique aiguë (FHF) provoquée par la galactosamine. Nous avons aussi étudié ces changements après hémoperfusion.

Nous avons mis en évidence une augmentation généralisée des concentrations en acides aminés dans le plasma sanguin systémique, le plasma portal, le CSF, les tissus du cerveau, du foie, des reins et des muscles striés. Les muscles striés constituent la plus importante source d'acides aminés accumulés lors d'insuffisance hépatique aiguë, les autres tissus contribuant aussi à l'accroissement des acides aminés dans le corps. Parmi tous les acides aminés, acides aminés branchés (BCAA) inclus, les acides aminés aromatiques (AAA) tyrosine, phénylalanine et tryptophane (libre et total) présentent la plus grande augmentation, et ce, dans tous les tissus étudiés. Le rapport molaire BCAA/AAA est significativement réduit dans tous les tissus étudiés. Le niveau de l'acide γ-amino-butyrique augmente de façon significative dans le cerveau et le tronc cérébral. Nous avons constaté que l'augmentation en tryptophane est dûe, pour une grande partie dans le cas du plasma et à presque 100% dans le cas du cerveau, à la forme libre, c'est-à-dire non liée à des protéines. L'augmentation en tyrosine dans le plasma est étroitement liée avec la tyrosine du cerveau. Un certain nombre d'acides aminés aromatiques dans le plasma voient leur niveau significativement réduit après hémoperfusion avec du charbon active recouvert de collodion (CAC). Il s'en suit une diminution significative des AAA dans le CSF, mais pas dans le cerveau, chez les rats atteints de GalN-FHF. L'hémoperfusion utilisant de la tyrosine immobilisée dans des cellules artificielles provoque une diminution sélective du niveau de tyrosine dans le plasma mais n'a pas d'influence sur le niveau de tyrosine dans le cerveau. Après hémoperfusion, le plasma se trouve clarifié en acides aminés aromatiques. Nos résultats suggèrent aussi une perte de la barrière sang-cerveau pour les acides aminés à travers les capillaires. Cependant, les mécanismes de transport entre les cellules du cerveau elles-mêmes et le liquide interstitiel semblent être maintenus.

Grâce à des chromatographies en phase liquide effectuées en série sur des échantillons de plasma et d'extrait de cerveau de rats atteints de GalN-FHF, nous avons pu mettre en évidence l'accumulation de métabolites de poids moléculaire moyen (MW 500-2000) traduite par l'augmentation du pic 7g des substances de poids moleculaire moyen. L'hémoperfusion CAC entraine une diminution significative du niveau de la fraction 7g à la fois dans les échantillons de plasma et d'extrait de cerveau. Nous avons démontré que la fraction du sous-pic 7g contient des substances peptidiques. D'autres études en électrophorèse sur gel de polyacrylamide SDS ont prouvé la nature peptidique de certaines de ces substances de poids moléculaire moyen. Le

poids moléculaire de ces peptides se situe entre 1,300 et 1,400. Des dosages radio-immunologiques montrent une augmentation de la concentration de la substance P (MW 1,345) dans le plasma des rats atteints de GalN-FHF.

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LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
α-ABA	Alpha-Amino butyric acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BBB	Blood brain barrier
BCAA	Branched chain amino acids
BW	Body weight
CAC	Collodion-coated activated charcoal
CPM	Counts per minute
CSF	Cerebrospinal fluid
dl	Deciliter
EM	Electron microscopy
FHF	Fulminant hepatic failure
GABA	Gamma-amino butyric acid
GalN	D(+)-Galactosamine
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
HPLC	High performance liquid chromatography
hr	Hour
Ile	Isoleucine
i-SP	Immuno-reactive substance P

Leu	Leucine
Lys	Lysine
2-ME	2-Mercaptoethanol
Met	Methionine
min	Minute
MM	Middle molecule
OPA	o-Phthaldialdehyde
Orn	Ornithine
p	Probability
Phe	Phenylalanine
PHU	Peak height unit
RIA	Radio-immuno assay
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
SMAC	Sequential multiple autoanalyzer with computer
Tau	Taurine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UDP	Uridine diphosphate
UV	Ultraviolet
Val	Valine
Vd	Volume of distribution

CHAPTER 1

INTRODUCTION

1.1. FULMINANT HEPATIC FAILURE (FHF): THE HISTORICAL REVIEW

1.1.1. Definition and General Description

Fulminant hepatic failure is defined as a clinical syndrome that occurs as a result of massive necrosis of liver cells in a patient who has had no previous evidence of liver disease (Trey & Davidson, 1970). The most prominent feature of this syndrome is the drastic onset of a progressive and severe encephalopathy, which is usually given the term "hepatic encephalopathy", or "hepatic coma". This syndrome is also characterized by a mortality rate which is as high as 80-90% in those who develop deep coma (Ritt et al, 1969; Silk & Williams, 1978). The high mortality is very closely related to the severity of hepatic encephalopathy. Although the survival rate is very low, FHF patients who recover do so completely due to the potential capacity of hepatocellular regeneration. Cirrhosis is rarely seen among survivors of FHF, in spite of the very severe and extensive initial liver necrosis. This is quite unlike liver failure exacerbated from chronic liver diseases which have very limited potential of hepatic regeneration.

The clinical causes of fulminant hepatic failure may be classified into 4 categories (Rueff & Benhamou, 1973). They are: (1) infections; (2) chemical intoxications; (3) ischemia and hypoxia; and (4) metabolic anomalies:

(1) Infections:

Viral hepatitis;

Marburg monkey disease;

Disseminated herpes simplex;

Virus infection and reovirus infection;

Coxsackie virus infection;

Adenovirus infection;

Glandular fever;

Q fever;

Others.

(2) Chemical intoxications (including poisons and drugs):

Amanita phalloides;

Paracetamol;

Tetracycline;

Yellow phosphorus;

hydrocarbons (halogenated);

Ethanol;

Halothane;

Metahexamide;

Monoamine oxidase inhibitors;

Hycanthone;

Others.

(3) Ischemia and hypoxia:

Ligation of hepatic artery;

Acute Budd-Chiari syndrome;

Acute circulatory failure;

Acute pulmonary failure;

Heat stroke; Others.

(4) Metabolic anomalies:

Acute fatty liver of pregnancy;

Reye's syndrome.

Others.

Among all these causes of human fulminant hepatic failure, the most common one is viral hepatitis. Williams (1976) reported 59 cases of FHF, among them 24 were due to virus A, 17 virus B, and 18 were unclassified. Apart from viral hepatitis, the causes of fulminant hepatic failure vary according to different epidemiological conditions. Paracetamol overdose is found to be an important etiological agent in causing FHF in the United Kingdom. Amanita phalloides intoxication is responsible for a considerable number of cases in France. Halothane-associated hepatotoxicity has been found in surgical patients in many countries. Besides, fatty liver of pregnancy may also cause acute liver failure (Saunders et al, 1979).

The liver is the site where most biochemical metabolism takes place. The failure of the liver in metabolizing carbohydrates, proteins, fat, nucleotides, and endogenous hormones may result in the following metabolic abnormalities (Duffy & Plum, 1982; Rueff & Benhamou, 1973; Silk & Williams, 1979; Anastacio et al, 1978; Zieve, 1979):

(1) Brain oxygen and glucose utilization are decreased;

(2) Affinity of hemoglobin for oxygen is reduced;

(3) Ammonia is increased in blood, muscle, brain, and

spinal fluid;

(4) Alpha-glutaramate and glutamine are increased in muscle, brain and spinal fluid;

(5) Pyruvate, lactate, citrate, and α-ketoglutarate are increased in blood and muscle, α-ketoglutarate, fumarate, malate, and oxaloacetate are decreased in the brain;

(6) Blood coagulation factors II, V, VII, IX, and X are decreased;

(7) Ketone production is decreased;

(8) Fatty acids are increased in blood;

(9) Amino acid patterns are altered in blood, brain, CSF and Urine;

(10) Brain concentrations of some neurotransmitters aspartate, glutamate, and norepinephrine are decreased;

(11) Neurotransmitter metabolites are increased in brain and CSF;

(12) False neurotransmitters are increased in blood, brain, muscle, and urine;

(13) Mercaptan concentrations are increased in blood, brain, urine and breath;

(14) Albumin levels are reduced in plasma;

(15) Phenolic compounds are increased in blood.

(16) Others.

These metabolic disturbances may be involved in the precipitation and progression of the syndrome of hepatic encephalopathy which is a consistent feature of fulminant hepatic failure. This syndrome is clinically characterized by

disturbances of consciousness; personality, and intellectual capacity as well as neuromuscular activity. As summarized by Plum and Hindfelt (1976), this syndrome consists of:

(1) Personality changes and impaired mental functions;

(2) Prominent motor abnormalities (Asterixis, tremor, paratonia, hyperactive stretch reflexes, and even decerebrate posturing);

(3) Alteration of consciousness;

(4) Characteristic neuro-ophthalmological changes, including intact pupils and brisk ocular responses;

(5) A constellation of laboratory abnormalities.

Among other criteria, the grading of severity of the consciousness deterioration is regarded as the most important indication of prognosis. The following is the summary of the classification being commonly used (Trey et al, 1966; Saunders et al, 1979; Silk & Williams, 1979):

Grade 1: Euphoria, occasional depression, fluctuant and mild confusion, shortened attention span, slowness of mentation, untidiness, slurred speech, disorder in sleep rhythm.

Grade 2: Accentuation of Grade 1, drowsiness, lethargy or apathy, inappropriate behavior.

Grade 3: Sleeping most of the time but arousable, incoherent speech, marked confusion.

Grade 4: Deep coma, unarousable, may or may not respond to painful stimuli.

Both fulminant and chronic liver failure may lead to hepatic coma when the severity of liver damage reaches a certain extent and the precipitating factors become influential. The question has been constantly asked whether hepatic encephalopathies caused by fulminant and chronic liver failure may share a common pathological mechanism. It is only known, that fulminant and chronic liver failures have different underlying lesions in the liver. FHF features an abrupt disseminated necrosis of the whole liver organ, while chronic hepatic failure is characterized by a disorganized liver parenchyma and a distorted hepatic blood circulation of hepatic artery and portal vein.

The major causes of death in patients with fulminant hepatic failure are: hepatic coma, cerebral edema, renal failure, congestive heart failure, hemorrhage secondary to lack of clotting factors and/or portal hypertension, and sepsis (Fischer, 1978). Among these factors, renal failure is usually functional, which occurs without an identifiable histological renal abnormality. Hepatic coma, brain edema and heart failure may advance from a reversible stage to the irreversible stage.

1.1.2. Liver Failure and Brain Dysfunction

The concept of the failing liver being responsible for the malfunctioning of the nervous system can be dated back to several thousand years ago. At about 1,000 BC, the ancient Chinese regarded the liver as the lodgment for the soul (Garrison, 1961a). The Babylonians considered the liver as the

soul because they thought it was the source of the blood (Garrison, 1961b). Both Hippocrates and Galen not only described jaundice and recognized its relationship to impaired liver function, but also noticed that intermittent delirium often occurred with jaundice (Adams, 1939; Singer, 1925).

In 1660, Rubeous made the earliest account of acute yellow atrophy and recognized that excitement and coma were often associated with jaundice (Brown, 1957a). A century later Morgagni made a most authentic description of acute yellow atrophy associated with delirium and convulsions (Brown, 1957b). Griffin, in 1834, succeeded in treating hepatic coma in an 11-year-old boy, using a therapy similar to today's treatment in rationale (quoted from Brown, 1970). The success of this therapy was attributed to the castor oil purgation of the guts. It was believed that such purgation removed toxins from the guts. Bright (1836) reported a case of acute fatal jaundice with mental and neurological disorders. The characteristic features he described of his case were similar to what we call hepatic coma today. His descriptions were, namely,dilatation of the pupils, irregular jerking movements of the extremities, dysarthria, confusion and delirium followed by coma and death. In 1858, Frerichs, in an analysis of 31 cases of acute yellow atrophy, noted the similarities of abnormal conditions of the nervous system shared by every case. Frerichs (1858) injected bile into the veins of dogs to determine its effects on the nervous system. He concluded that bile was ineffectual in inducing neurological symptoms, and not

responsible for the hepatic failure symptoms. But Kühne (1957), who performed similar experiments a century later, disagreed with this result and suggested that in fact bile was pathogenic.

Due to the animal experiments with Eck's fistula (Eck, 1877), much of the early research on hepatic failure was centered on the role of ammonia (Hahn et al, 1893; Balo & Korpassy, 1932; McDermott & Adama, 1954). It was believed that the principal defect in hepatic failure is in ammonia metabolism. Indeed, experimental results obtained using Eck's fistula seemed to support this theory. Ammonia in portal blood is derived from the colon and the small intestine owing to both protein breakdown and urea degradation. Ammonia is normally converted to urea in the liver by the Krebs-Henseleit urea cycle. Due to hepatonecrosis, blood ammonia is not adequately converted to urea even though the urea cycle enzymatic activities may still be maintained within normal range. The overproduction of ammonia in the gut further accentuates the accumulation of ammonia in systemic circulation.

In 1914, Chesney, Marshall and Rowntree (1914), published their observation that amino acids were elevated in hepatic failure. Soon after, Stadie and Van Slyke (1920) studied the terminal hepatic failure due to acute yellow atrophy and found an increase in tne urinary excretion of ammonia and titratable amino acids. These investigations marked the beginning of the research on amino acid disturbances in fulminant hepatic failure. The use of paper chromatography in medicine made the

analysis of amino acids in biological specimens feasible. The alteration of glutamic acid metabolism in the brain and liver was noted in the 1930s (Quastel, 1935; Weil- Malherbe, 1936). Later on, Walshe (1951) demonstrated the increase of glutamine in CSF of hepatic coma. The changes in pyruvic and lactic acid concentrations in the blood in hepatic failure were also observed (Amatuzio & Nesbitt, 1950).

Harper (1951), and Bessman (1958), postulated that elevated cerebrospinal fluid ammonia, secondary to an arteriovenous difference in ammonia concentrations but not to blood ammonia concentrations, caused hepatic encephalopathy. The observation was consistent with Walshe's theory that in the central nervous system excessive amounts of ammonia favour the glutamic acid to form glutamine (Walshe, 1951). Glutamine is thus built up in the brain, whereas glutamic acid is diminished in the brain. This will in turn reduce the brain reserve of alpha-ketoglutarate which is an important constituent of tri-carboxylic acid cycle. The diminution of glutamic acid and alpha-ketoglutarate will severely hamper energy supply for the brain.

It was discovered later that there was a wide range of ammonia concentrations at which toxicity occurs. This was explained by the changes in blood pH and serum potassium levels. At normal blood pH, more than 99% of blood ammonia exists as ammonium ion. As blood pH increases, the ionized ammonium tend to shift to molecular ammonia which is more toxic. This shift was postulated as a cause for neurological

disturbances in hepatic failure.

For a long time up to the 1960s, this "ammonia intoxication theory" had dominated in the field of hepatic failure research. Since then, progress in medical sciences and improvements in analytical techniques have led to new findings. As a result, a number of new theories on the fundamental metabolic disturbances in hepatic failure have been postulated.

The major theories will be reviewed in the next section (section 1.2.) of this thesis.

1.2. CONTEMPORARY CONCEPTS OF METABOLIC ABNORMALITIES IN FULMINANT HEPATIC FAILURE

1.2.1. Amino Acid Derangements and False Neurotransmitters

For many years in parallel with the theories of the disorder in ammonia metabolism was the observation that the amino acid concentrations were increased in blood and urine in fulminant hepatic failure patients.

It was known even a century ago that tyrosine metabolism was abnormal in patients with liver disease (**Rokitansky, 1855**). Tyrosine and Leucine crystals could be found in the urine of patients with acute yellow atrophy (**Frerichs, 1854**). In 1920, the increased titratable urinary amino acids were found in the terminal coma following acute yellow atrophy (**Stadie & Van Slyke, 1920**). Mann (1927) showed that even when concentration of the total amino acid nitrogen in the blood was within normal range, individual amino acids could still be toxic if present in excess. Hepatectomized dogs became

neurologically symptomatic after intravenous infusions of glycine or alanine which did not produce any symptoms in normal animals. Walshe (1953) studied 2 cases of massive hepatic necrosis and found a definite abnormal pattern of plasma amino acids with the most notable finding being the extremely elevated methionine level. Flock et al (1953), observed an increase of phenylalanine and tyrosine together with glutamine in the brains of hepatectomized dogs. In 1957, glutamine was found to be the overwhelmingly predominant amino acid in the brain and spinal fluid in the hepatectomized dogs.

Derangements of plasma amino acids in patients and animals with hepatic encephalopathy have been studied in some detail by Fischer's group (Fischer, 1974; Fischer et al, 1974; Fischer et al, 1975; Fischer, 1979; Fischer, 1982). The pattern is characterized by increased phenylalanine, tyrosine, free but not necessarily total tryptophan, methionine, histidine, glutamate and aspartate, together with decreased or normal levels of the branched chain amino acids leucine, isoleucine and valine. This finding has led Fischer to propose the "false neurotransmitter" hypothesis (Fischer, 1974). Fischer et al (1975) found that the molar ratio of branched chain amino acids (BCAA) to aromatic amino acids (AAA) is an important parameter indicating the severity of hepatic encephalopathy. This ratio is expressed as: Leu+Ile+Val / Tyr+Phe (BCAA/AAA). This molar ratio is significantly decreased in hepatic failure due to the drastic elevation of AAAs. Aromatic amino acids (AAA) and branched chain amino acids (BCAA) share a common carrier-

mediated transport mechanism across the blood brain barrier into the brain. The decreased BCAA competition at the blood brain barrier causes an increased influx of tyrosine and phenylalanine to the brain. Both Tyr and Phe are important precursors for the synthesis of neurotransmitters, the catecholamines. The enhanced cerebral influx of phenylalanine may competitively oversaturate and inhibit the enzyme tyrosinase hydroxylase which is normally the rate-limiting enzyme in converting tyrosine into the adrenergic neurotransmitters, dopamine and norepinephrine, in the brain. The excess cerebral tyrosine is then metabolized through a side-pathway to be decarboxylated to tyramine. Tyramine is further converted by beta-hydroxylation to octopamine, which has only very weak neurotransmitter action. When overproduced, octopamine may replace the normal adrenergic neurotransmitters and cause neural inhibition. Octopamine was thus named by some authors as a "false neurotransmitter". In a similar manner, accumulated phenylalanine in the brain is converted to beta-phenylethanolamine, which is also a "false neurotransmitter". These so-called "false neurotransmitters" are actually very weak normal neurotransmitters which have only 1/50 of the sympathomimetic activity of noradrenaline. They competitively replace the normal neurotransmitters with very weak functions in neurotransmission. When in excess, they can cause depression of cerebral function. Besides, the accumulated tryptophan in the brain causes an overproduction of serotonin, which is an inhibitory neurotransmitter (Fischer &

Baldessarini, 1971; Fischer, 1974).

The ultimate net effect of this series of events is the replacement of normal "excitatory" neurotransmitters by weaker ones or those with inhibitory effects.

This amino acid ratio and false neurotransmitter hypothesis has been extended recently to include ammonia in a unified hypothesis (James et al, 1979). In this composite theory, ammonia is converted to form glutamine in the cerebral pericapillary cells. Glutamine shares the common transport carrier with other neutral amino acids at the blood-brain barrier. The down-gradient efflux of glutamine from the brain consequently causes a great influx of aromatic amino acids from plasma into the brain by an exchange transport mechanism.

However a number of other studies did not support Fischer's theory. The postulated false neurotransmitter octopamine, when injected intraventricularly, failed to induce coma or consciousness changes in rats, even though the octopamine concentration in the brain was increased by 20,000-fold and an appreciable depletion of brain noradrenaline and dopamine did occur (Zieve & Olsen, 1977). In various parts of the brains of patients who died of hepatic encephalopathy, the concentrations of noradranaline and dopamine were unchanged and the level of octopamine was decreased (Cuilleret et al, 1980).

Likewise no significant changes in the adrenergic neurotransmitters could be demonstrated in patients with hepatic encephalopathy. The administration of L-dopa, an adrenergic neurotransmitter agonist, in these patients did not improve the consciousness recovery (Michel et al, 1980).

Lal et al (1974) studied the changes of CSF homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), lactic acid and pH in patients with hepatic coma or recently recovered from hepatic coma, and also in patients with miscellaneous neuropsychiatric disorders but without hepatic dysfunction. Their conclusion was that there was no change in dopamine or serotonin synthesis in liver failure patients when compared to patients with non-hepatic diseases.

A multicenter study, involving 5 medical centers and 50 patients with liver failure and acute hepatic coma, was recently reported by a number of investigators in Sweden and France (Wahren et al, 1983). The intravenous administration of branched chain amino acids in patients did reduce the concentrations of aromatic amino acids in plasma, but neither the improvement of cerebral function nor the decrease of mortality could be proven when the treated group was compared with the placebo group. They stated that the neurological dysfunction is not fully explained by the changes in plasma amino acids.

In Zieve's experiments (Zieve et al, 1980), dogs and rats were injected intravenously with Phe, Trp and Met solutions. Even though the concentrations of these amino acids in the

plasma and brain increased by 300 fold and 30 fold respectively, all animals remained alert during and at the end of the 6-hour infusion. These data seemed to disagree with the hypothetical key role of increased amino acids in the neurological disorder in hepatic failure.

In conclusion, the increase in blood and brain amino acid concentrations has been found to be related to the course of hepatic failure, but the pathogenic role of amino acid disturbance in hepatic failure and hepatic coma still requires further clarification.

1.2.2. Synergism Among Toxic Metabolites: Mercaptans, Fatty Acids and Ammonia

Zieve and his coworkers have proposed that the principal metabolic disturbance in FHF is caused by the synergistic effects of ammonia, mercaptans and fatty acids (Zieve & Nicoloff, 1975; Zieve, 1979; Zieve, 1980a). These three substances when administered individually into the experimental animals may cause neurological disturbances.

Ammonium has been shown, besides its interference to the tricarboxylic acid cycle and glutamate metabolism, to inhibit neuronal Na^+, K^+-ATPase activity by competing with K^+ (Schenker et al, 1967). Na^+, K^+-ATPase has a high concentration in the brain. It is responsible for the maintenance of transmembrane ion gradients which are necessary for the normal neuronal activity. Interference with this system may impair membrane repolarization and cause coma, as well as disturb the

functional integrity of the blood-brain barrier. High concentration of ammonium ions may partly substitute for intracellular Na^+ and block the outwardly- directed chloride pump. This can cause a shift of the inhibitory postsynaptic reversal potential toward the resting membrane potential and promoting central neurological inhibition.

Mercaptans may be produced by bacterial metabolism of methionine in the gut. Blood levels of methanethiol and dimethyl sulfide, have been found to be significantly increased in liver failure patients (Doizaki & Zieve, 1977; McClain et al, 1978). Blood levels of mercaptans correlated well with the degree of encephalopathy. Mercaptans are potent inhibitors of microsomal Na^+, K^+-ATPase activity, and their action occurs at several sites on the enzyme system (Quarforth et al, 1976). Methyl mercaptan has been isolated from the urine of the hepatic coma patients and is found in the breath along with dimethyl sulfide and dimethyl disulfide. The combination of all three is responsible for fetor hepaticus (Foster et al, 1974). In vivo animal experiments have shown that when the breathing air contained 0.16 percent methanethiol, the experimental rats developed reversible coma (Zieve et al, 1974).

Fatty acids, particularly short and medium-chain fatty acids, are known to accumulate in the blood in liver failure (Zieve & Nicoloff, 1975). Butyrate, valerate and octanoate are increased in the blood and CSF of patients with hepatic encephalopathy (Chen et al, 1970; Takahashi, 1963). In

experimental animals, administration of fatty acids in sufficient dosage caused reversible coma. Fatty acids at very low concentrations in vitro depress the activity of a variety of enzymes, including Na^+, K^+-ATPase of the brain microsomal membranes, and acetyl Co-A formation, which in turn depresses the citric acid cycle (Zieve, 1980b).

Zieve (1980) noted the analogy between Reye's syndrome and hepatic failure. In Reye's syndrome, abnormalities of fatty acids and ammonia are particularly prominent and the concurrent hypoxia and hypoglycemia may augment their toxicity. The synergistic interactions are probably of great importance in the pathogenesis of this syndrome. In fulminant hepatic failure, the concurrent accumulation of ammonia, mercaptans and fatty acids are also prominent metabolic abnormalities.

Zieve's concept of synergism between neurotoxins were based on some observations made in the animal experiments (Zieve, 1980). It was found that in normal rats the respective doses of the postulated neurotoxins such as: ammonia, mercaptans, or fatty acids, required to induce encephalopathy are reduced substantially by administering simultaneously a dose of one of the other two substances which would otherwise be insufficient to induce encephalopathy when given alone. In other words, any individual intoxicant among the three is potentiated by each of the other two, to induce neurological disorder, or coma. In combination, much smaller doses and blood levels are required to produce coma. Furthermore, fatty acids and mercaptans were found to cause a great increase in

blood ammonia. Normal rats become comatose when they are given ammonium, fatty acids and mercaptans in the levels comparable to those observed in rats with acute experimental hepatic coma due to massive ischemic hepatic necrosis.

There have been some disagreements on this theory of synergism among toxic factors. In a study by Record et al (1982), there was a poor correlation between blood and brain mercaptan levels and the stage of hepatic encephalopathy. Blood and tissue fatty acid levels do not correlate with the stages of hepatic coma (Zieve, 1981). It is not certain whether the encephalopathy induced by individual toxicants, or the mixture of, ammonia, mercaptans and fatty acids is identical to that occured in human fulminant hepatic failure (Jones, 1983). The biochemical and neurophysiological alterations due to these toxins are not yet conclusive.

1.2.3. Cerebral Abnormalities and Blood Brain Barrier Changes

A variety of morphological changes have been noted in the brain of patients and animal models of hepatic failure. Brain edema and blood-brain barrier impairment are the two most prominent features of the cerebral pathology in FHF.

Brain edema has been proven in autopsies of the patients who died in hepatic coma due to fulminant hepatic failure. Most brains inspected were swollen and showed signs of terminal transtentorial or medullary herniation (Hoyumpa et al, 1979). Gazzard and co-workers (1975a) also reported a very high incidence of cerebral edema in their autopsy series of FHF

patients. In their review, 105 patients died out of a total of 132 FHF patients with grade III or IV coma. Among many of the autopsied cases, the dura was found tense and the brain was swollen with flattening of the gyri. Herniation of the cerebellar tonsils was found in 12 out of the 96 autopsy-proven brain edema cases. Temporal lobe herniation was found in 8 cases. In another report of complete autopsy examination of patients who died of massive hepatic necrosis, 16 out of 32 cases were found to have cerebral edema and 4 of these had evidence of cerebellar or uncal herniation (Ware et al, 1971).

In a pig model of FHF, the elevation of intracranial pressure was revealed using a subdural pressure transducer (Hannied et al, 1979). Even though cerebral edema is well documented and generally recognized as one of the major cerebral disorders in liver failure, the underlying pathology of this condition has not been adequately understood. While intensive treatment of cerebral edema with dexamethasone, hydrocortisone and/or glycerol permitted a 50% survival rate in patients with Reye's syndrome, such therapy showed no effects in patients with FHF.

Microscopic examination of the brain in FHF revealed swelling and diffuse enlargement of the protoplasmic astrocytes. The precapillary vacuolation is pronounced in the cerebral white matter as well as in the cerebral and cerebellar grey matter. The dominating morphological feature in the brain of hepatectomy-induced FHF rats was cerebral edema (Livingstone et al, 1977).

Potvin et al (1984) recently reported their studies of the cerebral abnormalities in hepatectomized rats with acute hepatic coma. Under the electron microscope, they found a marked watery swelling of astrocytes adjacent to the capillary endothelium. This swelling seemed to begin in foot processes and then spread through the cell causing a massive accumulation of fluid in astrocytes. Such change was found diffused in the grey matter of cerebrum, cerebellum, and in the putamen and globus pallidus. Astrocytic changes can also be reproduced in the experimental hyperammonemia. When examined with electron microscopy, cytoplasmic hypertrophy with marked proliferation of mitochondria and endoplasmic reticulum was demonstrated in early experimental encephalopathy. This was believed to reflect heightened metabolic activity in the astrocytes associated with ammonia detoxification (Norenberg, 1977).

In contrast to the astrocytes, the neuron cells show relatively minor alterations. This combination is specific for the liver diseases, for they are not observed in many other conditions, including cerebral anoxia, head injury or hypoglycemia.

As Finlayson (1982) pointed out, in the acute liver diseases, such as FHF, the major cerebral lesions are the diffuse and non-specific swelling and Alzheimer type II nuclear change of astrocytes. These changes are different from those in the chronic liver diseases, such as cirrhosis. In the chronic liver diseases, the brain lesion may appear to be irreversible hepatocerebral degeneration with neuronal loss and

"spongy" degeneration.

During the last decade, more attention has been directed to the possible impairment of blood brain barrier in liver failure. Livingstone et al (1977) reported the breakdown of the blood-brain barrier in acute hepatic coma in hepatectomized rats. When rats were in late stage 3 or stage 4 hepatic coma, after a two-stage hepatectomy, the blood brain barrier was found permeable to D-sucrose, inulin, and L-glucose, all of which are normally excluded by the barrier. The brains of the comatose rats were stained by trypan blue while normal control brains were not. The authors also found that the formation of brain edema was associated with the blood brain barrier breakdown.

When acute hepatic coma was induced in rats by 2-stage hepatectomy, the electron microscopic examination of the brains revealed the appearance of greatly increased numbers of vesicles in the capillary endothelial cells. The passage of horse radish peroxidase, as a permeability tracer, was found markedly increased and the tracer was occasionally found in the capillary basement membrane. The experimentation with low molecular weight microperoxidase achieved same results. The formation of capillary endothelial vesicles was suggested to be related to cerebral edema.

In a rabbit model of acute liver failure induced by galactosamine, a general, non-specific increase of 5 to 10 fold in brain capillary permeability was demonstrated (Horowitz et al, 1983). The study using ^{14}C-alpha-amino-iso-butyric acid, a

marker of brain capillary permeability, convincingly demonstrated that the increase in brain capillary permeability preceded the onset of overt hepatic encephalopathy. Therefore, the permeability change of the blood brain barrier may be one of the major contributing factors in acute hepatic encephalopathy.

In two animal models of fulminant hepatic failure, i.e., galactosamine hepatitis and hepatic devascularization, there was a 3-fold increase in the passive permeability of the blood brain barrier to inulin and sucrose (Zaki et al, 1984). Transport of amino acids also significantly increased by 30% to 65%. Dixit and Chang (1984) found the progressive development of cerebral edema and the blood brain barrier breakdown during the course of GalN-induced fulminant hepatic failure and coma. It has been postulated that the circulating toxic substances in FHF may be responsible for such changes in blood brain barrier permeability. Recently the investigation by Williams' group has shown that classical toxins of hepatic failure, such as ammonia, methyl octanoate, mercaptans and phenol, when administered intravenously, intraperitoneally, or by inhalation to the normal rats, caused significant increase in blood brain barrier permeability (Zaki et al, 1983). The quantity of each toxin injected was calculated so that the resultant plasma concentration was approximately the same as the pathological levels seen in patients with FHF. Escherichia coli endotoxin infused into rats following partial hepatectomy also increased blood brain barrier permeability.

Some authors, however, have reported negative findings on blood brain barrier changes (Crinquette et al, 1978; Herlin et al, 1981). The difference in the animal models used may partly account for such discrepancy. The model of portocaval shunt in the rat, for example, does not show any increase in blood brain barrier permeability to horseradish peroxidase when observed 10 to 30 days after the operation. (Laursen & Westergaard, 1977).

In conclusion, brain edema and blood brain barrier impairment are the two major features of the cerebral abnormality in FHF. The causes of these abnormalities are probably the cytotoxic effects of the circulating toxins on the blood brain barrier structures.

1.2.4. Disorder in GABA Metabolism

In comparison to the other theories of neurochemical and neuropathological abnormalities, the theory of GABA being pathogenic in liver failure is relatively new, but appears very plausible. GABA (gamma-amino butyric acid) is the principle inhibitory neurotransmitter in the mammalian brain. Some 25% to 45% of all nerve endings in the brain are GABA-ergic. After being released from presynaptic storage sites, GABA binds to postsynaptic receptors, resulting in increased chloride ion flux through the neuronal membrane and subsequent hyperpolarization (inhibition) of the postsynaptic neurons.

Schaefer and Jones (1982), first proposed the concept of neuroinhibition by GABA in hepatic encephalopathy. Using a rabbit model of FHF coma induced by galactosamine, they showed

that intestinal GABA was not metabolized by the damaged liver. The level of GABA in the portal plasma was about twice that in the aortic plasma. The development of hepatic encephalopathy was associated with increased levels of GABA in plasma and increased blood brain barrier permeability to GABA. In the brain, the numbers of GABA binding sites were increased.

Thus, the hypothesis is that in liver failure gut-derived GABA passes through a permeable blood-brain barrier and induces its own receptors in the brain, and that gut-derived GABA contributes to the neural inhibition of hepatic encephalopathy.

The specific binding of ^{3}H-GABA to postsynaptic neural membranes was studied in membranes prepared from normal brains and brains obtained during hepatic coma. Computer analysis of the binding data indicated the presence of two independent GABA receptors. The affinities of both receptors remained unchanged, but the numbers of both GABA receptors in the hepatic coma brains were found almost doubled. No increase in receptor number was seen in normal control or terminal uremic coma. A phenomenon of up-regulation of GABA receptor was suggested. Such up-regulation was induced by gut-derived GABA which "invaded" the brain to reach postsynaptic binding sites. The receptor up-regulation may increase the sensitivity of the brain to GABA-ergic neural inhibition. In patients with hepatic coma, the apparent enhanced sensitivity to barbiturates and benzodiazepine may be explained by the increased number of GABA receptors. These drugs exert their effects via the GABA receptor complex which is believed to be the common site for

both GABA and the tranquillizers (Paul et al, 1981).

In a rabbit model of hepatic failure induced by galactosamine, coma was consistently associated with a series of distinctive changes in the visual evoked potential waveform (VEP). VEP is considered as a proper measurement of the electrical activity of nervous tissue in response to stimuli. It reflects excitatory post-synaptic potentials, inhibitory post-synaptic potentials or some mixture of both (Purpura, 1972). The pattern of the visual evoked potential in hepatic coma was identical to that in coma induced by three drugs which activate GABA-ergic neural mechanisms: pentobarbital, benzodiazepine, and muscimol. The VEP patterns induced by these drugs differ fundamentally from those of ether-induced coma, acute hyperammonemic encephalopathy, postictal coma, and toxin-induced coma resulting from the administration of a combination of three neurotoxins: ammonia, dimethyl disulfide, and octanoic acid (Pappas et al, 1984).

It has been cautioned that identical VEP waveforms in different neurologic states do not constitute unequivocal proof that different syndromes share common neural mechanisms. However, these findings did imply an increased sensitivity of the GABA neurotransmitter-receptor system in hepatic coma. The abnormal VEP patterns in hepatic coma can be reproduced by administering drugs that induce activation of the GABA neurotransmitter system.

This GABA theory is now under further study and has attracted much research interest.

1.2.5. Lack of Essential Factors

While most of the recent studies have focused on the accumulation of toxic metabolites, it should not be neglected that the lack of essential substances is also one of the major contributing factors in the general metabolic abnormalities in fulminant hepatic failure. The normal liver produces a great many substances that are released into the circulation. Some of these substances are essential for the normal functioning of other tissues including the brain.

Glucose, for example, is essential for brain function. It is nearly the only source of energy the brain can use. Hypoglycemia results from the inability of the necrotic hepatocytes to produce glucose from glycogen or by gluconeogenesis. The insufficient degradation of the circulating insulin by the failing liver can further decrease blood glucose level due to the increased peripheral glucose utilization.

The reduced synthesis of albumin results in a decrease in plasma albumin level. The degradation of albumin due to the general catabolism further accentuates this decrease. Therefore, there is a tendency for plasma albumin level to decrease in fulminant hepatic failure. Albumin normally functions as a carrier for a variety of hydrophobic or lipidophilic substances, e.g., bilirubin, fatty acids, etc.. The need to bind fatty acids and bilirubin requires a sufficient replenishment of albumin to compensate for the

decrease in blood level in liver failure.

The reduction of essential blood proteins other than albumin is also quite severe in FHF. The bleeding diathesis in FHF or chronic liver failure is known to be caused by the reduced blood levels of clotting factors II, V, VII, and probably IX and X, all of which are normally synthesized by the liver (Davidson, 1979). Thrombocytopenia, prothrombin deficiency expressed as prolonged prothrombin time, and depression of plasma fibrinogen are also constant features of fulminant hepatic failure (Rake et al, 1970). Deficiency in the clotting factors is generally attributed to reduced synthesis by the damaged liver, but in at least 1/3 of FHF patients utilization of these factors is also accelerated and is related to intravascular coagulation.

The normal brain function requires many essential substances supplied by the liver. It has been experimentally shown that the perfused cat brain functions better when the liver is included in the perfusion circuit than if the liver is excluded. Similar to this experiment, addition of fresh liver extracts to the perfusing medium without the participation of the liver in the circulation, also tends to normalize the metabolism of the brain. The addition of cytidine and uridine to the perfusate had a similar effect, suggesting that these are the two of the essential metabolites which the liver makes available to the brain (Geiger & Yamasaki, 1956). A Japanese group also reported the beneficial effects of cytidine and uridine administered to patients with severe liver diseases

(Takahashi et al, 1961). Dixit and Chang (1981) reported that a water soluble liver extract prepared from healthy young rats, when administered intraperitoneally in fulminant hepatic failure rats, significantly increased survival time in treated rats. This liver extract was also shown to significantly reduce gastrointestinal bleeding in these rats.

It was postulated that the water soluble liver extract must contain some of the essential factors which the normal liver produces to maintain the normal metabolism of the brain. It has been suggested that these "essential stimulatory substances" may be glycoproteins with molecular weights between 30 and 50 thousand daltons (Goldberg et al, 1980). However, The exact identity of these vital factors still needs to be established.

1.3. FULMINANT HEPATIC FAILURE ANIMAL MODELS

1.3.1. Requirements of Ideal FHF Animal Models

Many problems encountered in experimental and clinical medicine have been clarified by the proper use of animal models. The animal model simulation of clinical conditions has numerous advantages in that most of the factors which enter into the production and progression of abnormal processes are capable of exact control.

Although it is now possible to produce experimentally most of the abnormal conditions associated with hepatic failure in human beings, the development of an ideal animal model of

fulminant hepatic failure is still very difficult. This is due to the very complex nature of the disease, and also due to the marked variation among different species in response to hepatic injury. The historical experiment of Eck's fistula was probably the earliest trial of animal modeling in studying metabolic disturbances in liver diseases.

It is important to define the requirements of a suitable animal model of fulminant hepatic failure. The following requirements are generally considered to be fundamental and indispensable (Terblanche et al, 1975):

(1) Comparability: The liver lesion and the mortality of the animal model should be comparable to those of human fulminant hepatic failure. A selective liver lesion should be produced which gives rise to death from liver failure. A suitable time interval before death is required for experimental investigations. The total mortality should be comparable to the human counterpart.

(2) Reversibility: The hepatic failure produced should be potentially reversible, in order to enable animals to respond to experimental treatment.

(3) Reproducibility: The biochemical, pathological and neurological alterations, as well as the mortality rate, should be consistent through the experimental groups of the same model.

(4) Safety to personnel. Any drugs, techniques or microorganisms used should be of minimal hazard to laboratory personnel.

1.3.2. Anhepatic Models

Mann and Magath (1921) reported a dog model of total hepatectomy performed by a three-stage procedure. The first stage consisted of a lateral anastomosis of the portal vein and the vena cava between the entrance of the right lumbo-adrenal vein and the hepatic veins. This step was to develop the collateral circulation through the azygos and the internal mammary veins. A few weeks later, in the second stage, the portal vein was ligated at its entrance into the liver. This caused all blood from the viscera and hind legs to return to the vena cava by the way of collateral vessels. At a third operation at a later period, total extirpation of the liver was performed. The animal from which the liver was removed by this technique recovered from the immediate effects of the operation and then presented a syndrome comparable to what is seen in acute liver failure. Following total removal of the liver, the animal appeared normal for 5 to 8 hours, then developed muscular weakness and became perfectly flaccid and comatose. The animal finally died in severe convulsions.

This technique was later simplified by subsequent workers to a one-stage procedure where total hepatectomy was carried out with a side-side portocaval anastomosis, but without interruption of the inferior vena cava (Starzl et al, 1959; Serrou et al, 1971).

The hepatectomized animal model was not only established in the dog, but also in the rat. Flock's group (1966) reported

the techniques of two-stage hepatectomy in rats. An eight-week interval was allowed to enable development of adequate collateral circulation. Two years after Flock's report, a simplified two stage hepatectomy in the rat was reported (Bollman & Van Hook, 1968). The use of anhepatic animal models, whether in the dog or the rat, has definitely contributed to the research and the understanding of the metabolic, hemodynamic and neurological changes occurred in liverless status. Roche-Sicot et al (1974), used this hepatectomised rat model for studying the effect of cross-circulation on acute hepatic encephalopathy in the rat. Livingstone et al (1977) used the anhepatic rat model to investigate the changes in the blood-brain barrier in hepatic coma.

Potvin et al (1984) studied cerebral abnormalities in acute hepatic coma using this hepatectomized rat model.

Degos and his co-workers (1974) prepared their hepatectomized rat model in a three stage procedure which is different from the others'. This includes: (1) ligation of inferior vena cava; (2) three weeks later, end-to-side portacaval anastomosis; (3) 2 days later, removal of the liver. Using this model, they studied the electro encephalopathic changes in comparison with the eviscerated rats. They suggested that ammonia, and gut-released substances in general, play no part or at most a minor part in the mechanisms of hepatic encephalopathy of the liverless rat. The reason for this hypothesis was that the electroencephalopathic changes in

liverless rats were not different from that in eviscerated rats.

There have been some controversies with regard to the use of total hepatectomy as a suitable model for hepatic failure. The major disagreement was that this model does not meet the requirement of reversibility, and that the absence of damaged liver cells makes the model fundamentally different from human fulminant hepatic failure (Terblanche et al, 1974; Abouna et al, 1976).

To avoid such drawbacks, partial hepatectomy (67%, or, 2/3) was performed in some laboratories to establish rat models of fulminant hepatic failure (Ryan et al, 1980; Higgins & Anderson, 1931). Their results demonstrated that the capacity for a regenerative hyperplastic response following partial hepatectomy was retained. They also reported the suitability of this model to be used in the evaluation of the artificial liver support systems.

It is now generally agreed that partially hepatectomized animals may make good models for research on the mechanism and kinetics of hepatocellular regeneration and proliferation as a response to experimental injury of the liver (Yager et al, 1973; Widmann et al, 1973; Stocker and Wullstein, 1973; Gerhard, 1973).

1.3.3. Devascularization or Ischemia Models

Complete hepatic devascularization may produce non-reversible hepatic failure animal models. Several

devascularization techniques have been reported. Interruptions of hepatic artery or portal vein, or both, have been experimented on by a lot of centers. Most of the hepatic devascularization procedures involve, as a basic step, the famous Eck's fistula, which is the portal-systemic anastomosis first established by and named after the Russian physiologist Nikolai Vladimirovich Eck (Eck 1877). However, portacaval anastomosis technique when used alone produces only animal models of cirrhosis or portal systemic encephalopathy, but not models of fulminant hepatic failure.

There have been some efforts in combining other techniques with portacaval anastomosis to create acute hepatic ischemia animal models. Wustrow et al (1981) achieved an acute ischemia model in the young pigs by the operations in two steps: a portacaval shunt followed by a hepatic artery ligation within 24 hours. They studied the changes in plasma hormones such as insulin and glucagon, amino acids, ammonia and brain biochemistry. Nevertheless, despite significant elevation in blood ammonia, fatty acids, brain tissue glutamine, ammonia and tryptophan, the experimental animals remained awake and alert, and indistinguishable from sham-operated controls. Therefore this model did not resemble the fulminant hepatic failure in human which is usually accompanied by the neurological deterioration, ie., hepatic coma.

The models of such complete hepato-devascularization were found unsuitable for certain research purposes. Some researchers therefore proposed a temporal devascularization

after portacaval anastomosis. One good example was the modification of the classical canine model which was established in 1953 involving a two-stage procedure (Rappaport et al, 1953; Giges et al, 1953). In the original procedure, a permanent ligation of hepatic artery was performed some days after a portacaval shunt. Misra and associates (1972) modified the procedure in which the ambulant, fully conscious dog has a graded period of total ischemia to the liver. A temporal devascularization of the liver was achieved using the silicone rubber balloon occluders which were placed on the hepatic artery and portal vein after the completion of the portacaval shunt. The tests of hepatocytic function, hemodynamic status and metabolic, respiratory and reticuloendothelial function have been found to simulate those described in clinical fulminant hepatic failure. Battersby and co-workers (1974) performed similar experiments in the pig but using different techniques to temporarily devacsularize the liver at normal body temperature.

Abouna's group (Abouna et al, 1976). created a satisfactory model of acute hepatic failure by performing an end-to-side portacaval anastomosis followed 24 hours later by a temporal 1 hour occlusion of common hepatic and gastroduodenal arteries.

This type of tempory devascularization was believed to be able to generate a satisfactory animal model for fulminant hepatic failure (Terblanche et al, 1974).

1.3.4. Hepatotoxicant Models

1.3.4.1. Carbon tetrachloride model

The potent toxicity of carbon tetrachloride (CCl_4) to the liver was recorded as early as half a century ago (Lamson & Wing 1926; Bollman & Man, 1931). CCl_4 was extensively used as a solvent for waterproof airplane paint before it was discovered that many workers exposed to this poison contracted subacute yellow atrophy or cirrhosis. CCl_4 had also been used as a drug for the treatment of hookworm. This chemical has been injected into the animals to study the in vivo toxicity and the consequent pathological process which lead to hepatic failure. Animal models of CCl_4-induced hepatic failure have been studied in the rat (Cameron and Karunaratre, 1936; Forbes and Outhouse, 1940); the monkey (Trey et al, 1969); and the pig (Terblanche et al, 1974).

The mechanism of hepatic necrosis induced by CCl_4 was based on the metabolism of this substance in the liver, the development of a block in protein synthesis and the onset of fatty infiltration (Dianzani & Gravela, 1974). The CCl_4 intoxication causes liver damage in two stages: (1) marked lobular degeneration and necrosis of the liver which represents features of fulminant hepatic failure; (2) a cirrhosis stage, with proliferation of unaffected hepatocytes, which resembles cirrhosis and chronic hepatic failure in humans.

Although the early studies mostly centered on inducing

cirrhosis by the long-term, repeated administration of CCl_4 (Cameron and Karunaratne, 1936; Bollman and Man 1931), in recent years, CCl_4 has been used more frequently as a means of creating fulminant hepatic failure model. Trey and co-workers (1969) produced a fulminant hepatic failure model in the rhesus monkey by intravenous injection of CCl_4 through an arcuate mesenteric vein, a branch of the portal vein, at a dosage of Ø.18 ml CCl_4/kg body weight. They found that the clinical signs of fulminant hepatic failure described in man or in hepatectomized animals were all seen in their monkeys. They concluded that this model was reproducible, reversible and suitable for their studies on the effects of exchange blood transfusion on fulminant hepatic failure. Experiments done by Terblanche's group (1974) on CCl_4-induced hepatic failure model in the pig also favoured the use of this chemical as a suitable means in animal modeling. The injection of CCl_4 via hepatic artery, or portal vein in the pig produced a "reasonably satisfactory animal model", as commented by the authors themselves.

Even though some progress was achieved in CCl_4-hepatic failure animal models, the attempts to determine optimal dosage and optimal route of administration, have not met with success (van Leenhoff et al, 1974). A large number of animals are needed. Strictly controlled paired conditions are required before an objective and accurate evaluation can be made for this CCl_4-induced animal model.

1.3.4.2. Paracetamol model

Paracetamol (N-acetyl para-amino phenol, or, acetaminophen) has been used as an antipyretic and analgesic since last century. It is relatively safe in therapeutic dosage, but overdosage may cause liver damage, and even death in severe cases (Prescott et al, 1971).

In the animal experiments, acute overdosage of paracetamol in the rat (3g/kg, orally) causes liver necrosis in about 18 hours (Mclean, 1974). But it was found that the liver damage was very unusual in its extreme biological and toxicological variabilities. Under the same experimental conditions, some animals will have no liver injury, some have massive necrosis of all lobes, and some will have necrosis of only one lobe while parts of the lobe or some of the other lobes remain uninjured. Thus, the histological and pathological assessments seem difficult and the use of this model still needs to be justified (Gazzard et al, 1975b).

It was probably because of the same reason that the paracetamol model in the pig was rejected by Terblanche's group after experiments with three groups of pigs (Terblanche, 1974). The mean survival time was quite variable, the dose-response was unpredictable, and the reproducibility unsatisfactory. In addition, an unexplained acute anemia occurred in a significant number of animals, making the clinical signs more complicated.

These problems clearly indicated the unsuitability of paracetamol intoxicated animals as a model for fulminant

hepatic failure.

1.3.4.3. Other chemical induced animal models

A number of other chemicals have been used for producing animal models of fulminant hepatic failure. Most of these chemicals exert their toxic effects on the liver while they are enzymatically metabolized and removed by hepatocytes, more specifically, by microsomes.

(1) Anesthetics. The anesthetic halothane has been used after phenobarbital pretreatment in rats to produce severe massive liver necrosis (Stenger & Johnson, 1972). Phenobarbital is known to induce microsomal enzymes which increases the metabolism of anesthetics. Consequently, the hepatotoxicity of the anesthetics is greatly enhanced by the co-administration of phenobarbital. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) administered this way has been associated with abnormal hepatic lipid accumulation, structural and functional alterations of liver mitochondria, reduced bromosulphthalein clearance, depression of hepatocellular enzyme systems and a significant reduction of hepatic microsomal cytochrome P-450 levels.

Fluroxene (2,2,2-trifluoroethoxy-ethene) was originally introduced into clinical anesthesia practice as long ago as 1953 (Kranz et al 1953). Fluroxene anesthesia after phenobarbital induction in the rat has been shown to cause a rapidly lethal hepatic necrosis (Harrison and Smith, 1973). Experimental rats died during or immediately after their

repeated three 1-hour exposures to fluroxene. Autopsies showed enlarged liver mass, extensive central and midzonal necrosis. Fluroxene administration alone, without phenobarbital induction, caused histologically detectable changes in the liver.

(2) Furosemide, or 4-chloro-N-furfuryl-5-sulfamoyl anthanilic acid. This is a commonly prescribed diuretic. In recent years, this drug was found to be associated with considerable hepatotoxicity which was mediated by a toxic metabolite. The administration of large doses of furosemide in male mice produced massive hepatic necrosis at 2-3 hour in midzonal hepatocytes and by 6 hour in centrilobular hepatocytes (Mitchell et al, 1973; Potter et al, 1973). Unfortunately, besides the hepatocellular injury, this drug also caused marked renal tubular necrosis. Such severe complications prevent this drug from producing an ideal model of fulminant hepatic failure.

(3) Dimethylnitrosamine (DMNA) Barnes and Magee (1954) first reported that DMNA, which is selectively toxic to the liver, produced acute centrilobular necrosis of the liver in the rat, mouse, guinea pig, rabbit and dog when given in doses of the order of 25 mg/kg body weight. This drug has been administered orally to dogs to induce cirrhosis (Madden et al, 1970). The consequent biochemical, physiological, and hepatohistological abnormalities resembled many features of hepatic cirrhosis in man. Experiments in 1972 by Kuster and Woods (1972) demonstrated that DMNA given intravenously in a

single dose of 30 μl/kg body weight was uniformly lethal to dogs with survival time of more than 24 hours. This model was found reproducible when the dose was properly controlled. The authors also found this a satisfactory animal model for the experiments on auxiliary heterotopic liver transplantation as a temporary support in hepatic failure. This model was also a model for cirrhosis (Madden et al, 1970). The suitability of this model for fulminant hepatic failure still requires more animal experiments and critical evaluations.

(4) Yellow phosphorus. Abouna and his colleagues reported a limited animal trial using yellow phosphorus by subcutaneous injection in the dogs to produce acute hepatic necrosis (Abouna et al, 1976). The technique was simple, the mortality was high and the mean survival time of 3.7 days was ideal. However, it suffers a number of drawbacks such as: the involvement of renal tubule injury; the poor predictability of the occurrence of hepatic coma; and the lability of the chemical to oxidation, etc.. Above all, this chemical is very hazardous to the laboratory personnel. Therefore, this model is far from being satisfactory.

1.4. GALACTOSAMINE-INDUCED FULMINANT HEPATIC FAILURE IN RATS: THE ANIMAL MODEL USED IN THIS PROJECT

1.4.1. General Description of Galactosamine (GalN) Hepatotoxicity

Ever since the first observation of the hepatotoxicity of galactosamine (GalN) in 1968, this aminosugar has been increasingly used as a means of inducing fulminant hepatic

failure animal models (Keppler et al, 1968). These animal models feature acute massive hepatic destruction; potential of hepatocytic regeneration; and similarities shared with human fulminant hepatic failure in histological, biochemical and pathophysiological alterations. The injury to the liver by GalN is reproducible, and reversible. This chemical exerts its toxicity very selectively on the liver with almost no involvement of other organs. There have been a great deal of basic studies reported in literature since the last decade with respect to the process of the biochemical abnormalities in the galactosamine-injured liver cells (Decker and Keppler 1972; Reutter et al, 1973; Pickering et al, 1975; El-Mofty et al, 1975; Chirito et al, 1977; Chang et al, 1978; Liehr et al, 1978; Chirito et al, 1979).

Galactosamine is the 2-amino-2-deoxy derivative of galactose. In 1967 it was accidently discovered that rats receiving D-GalN intraperitoneally suffered a rapid loss of liver glycogen (Keppler et al, 1968). The accompanying abnormalities were the reduction of plasma proteins including coagulation factors, and the increase of the activities of liver specific enzymes in blood plasma. Light microscopic examination of these livers revealed histological features resembling those seen in viral hepatitis. Subsequent studies of this chemical revealed the sequence of metabolic events that comprise the biochemical lesion of this liver injury.

D-GalN, although being a constituent of many heteroglycans, is normally not detectable as a free sugar in

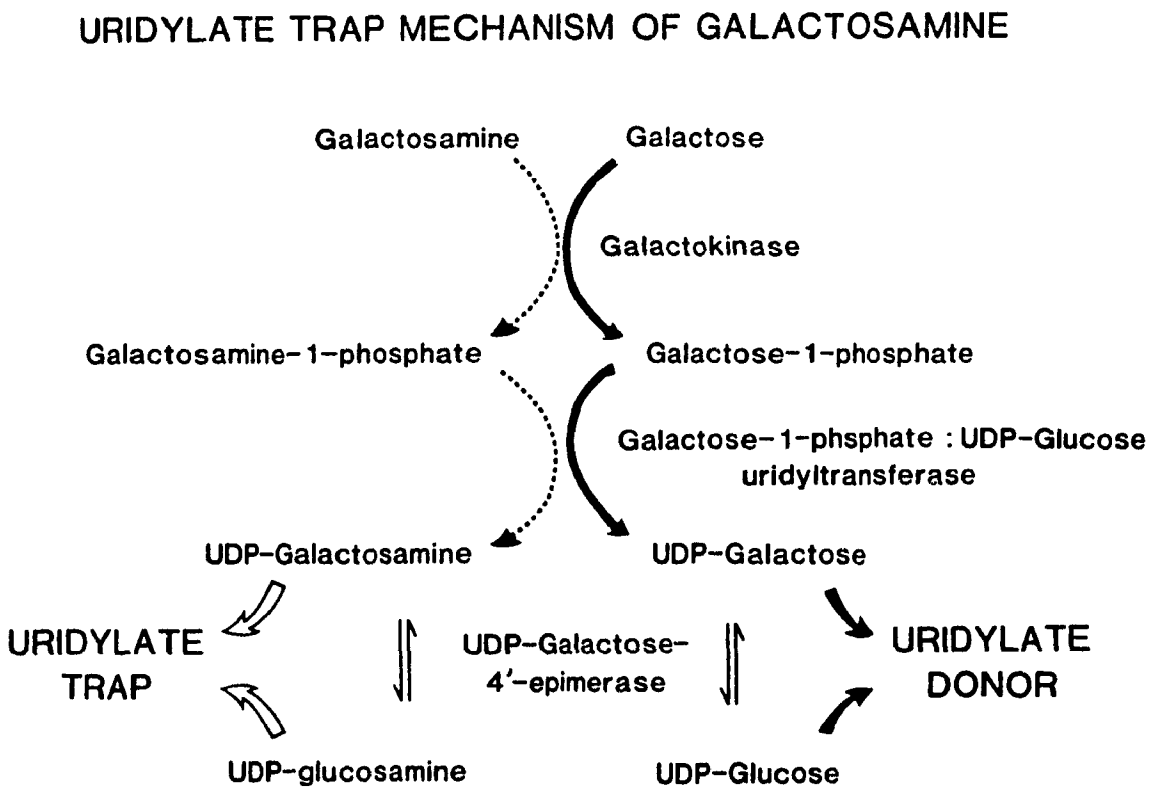


Fig. 1.4.1. The mechanism of GalN hepatotoxicity. Galactosamine competes with galactose for the enzymes galactokinase and galactose-1-phosphate:UDP-glucose uridyltransferase to form UDP-galactosamine which is then converted to UDP-glucosamine by UDP-galactose-4'-epimerase. Both UDP-galactosamine and UDP-glucosamine serve as the uridylate trap. The normal uridylate donors UDP-galactose and UDP-glucose are diminished.

normal animals. This amino sugar is known to be readily taken up and metabolized by the liver. About 20% of injected GalN (1.74 mmol/kg) was recovered from the liver 3 hours later (Decker & Keppler, 1972).

The biochemical mechanisms of GalN hepatotoxicity have been studied in detail (Decker& Keppler, 1972; Pickering et al, 1975; El-Mofty et al, 1975; Gool et al, 1978; Liehr et al, 1978). It has been concluded that GalN in excessive amounts serves as a uridylate trap causing depletion of UTP and UTP-glucose. As a result of this depletion, the syntheses of glycoproteins, glycolipids, and glycogens are severely hampered. This is the biochemical basis of the induction of GalN hepatitis.

Intravenous administration of radio-labelled ^{14}C-galactosamine have conclusively demonstrated the hepatospecificity of the drug. Virtually 100% of the injected GalN is removed from the circulation and is sequestered in the liver within 2 hours of infusion (Blitzer et al, 1978).

The schematic diagram of fundamental biochemistry is shown in Fig. 1.4.1.. Under normal circumstances, the hexose galactose is converted into galactose-1-phosphate through the enzymic reaction of galactokinase. Galactose is converted into UDP-galactose by the enzyme UDP-glucose:galactose-1-phosphate uridylyl transferase. Finally, UDP-galactose is converted to UDP-glucose by UDP-galactose-4'-epimerase. UDP-glucose (UDP-G) and UDP-galactose normally function as the uridylate donors and

are critically important to the UDPG-linked syntheses of glycogen, heteropolysaccharides and glucuronides (Decker et al, 1971).

As can be seen from Fig. 1.4.1.,when an excessive amount of GalN is administered into the animal and reaches the liver, it may compete against galactose for the enzymes in the synthesis of UDP-galactose and UDP-glucose. Consequently, GalN-1-phosphate and UDP-GalN gradually accumulate. Both substances have been identified as the predominant early metabolites of GalN in rat liver (Keppler & Decker, 1969). The conversion of GalN-1-phosphate to UDP-GalN is catalyzed by UDP-glucose:galactose-1-phosphate uridyl-transferase. However, this enzyme has a very low affinity for galactosamine-1-phosphate, thus causing the buildup of this compound. At about 1 hour after administration of GalN, maximal level of GalN-1-phophate is reached. The accumulation of GalN-1-phosphate is found to inhibit the enzyme UDP-glucose-pyrophosphorylase. This enzyme is responsible for catalyzing the reaction of glucose-1-phosphate and UTP to form UDP-glucose, with the by-production of a phosphate. GalN-1-phosphate competitively inhibits the UDP-glucose-pyrophosphorylase reaction. By these mechanisms, GalN-1-phosphate severely impaired the synthesis of UDP-glucose. UDP-GalN is converted by UDP-galactose-4'-epimerase to UDP-glucosamine. The latter compound apparently does not serve as a uridylate donor in the uridyltransferase reaction as does UDP-glucose which is regenerated in galactose metabolism. Therefore, the formation of UDP-GalN and UDP-

glucosamine functions as a trapping mechanism for uridylate. Due to the uridylate-a trapping mechanism of the GalN-induced chain reaction, the uridylate donors, UDP-glucose and UTP are heavily depleted. The drastic depletion of uridine triphosphate (UTP) and UDP-glucose in rat liver occurs as rapidly as in only 15 to 30 minutes. An inhibition of UTP- and UDP-glucose-dependent biosyntheses of glycogen, glucuronides, heteropolysaccharides, is considered as an important step leading to hepatocellular damage by galactosamine (Decker et al, 1971).

Based on the same principle, not only D-GalN, but also 2-deoxy-D-galactose (in combined action with 6-aza-uridine) may provoke the hepatitis-like liver damage (Decker et al, 1971). The formation and accumulation of UDP derivatives of these sugars function as a uridylate-trap mechanism, causing a marked depletion of UTP, UDP, UMP, UDP-glucose and UDP-galactose. Such a selective trapping of uridylate would be without cytotoxic effects if a level above a certain threshold of UTP or UDP-glucose is maintained, or if a low UTP level exists only for a short period of time. A uridine phosphate level of 30% of normal is a putative limit which fits best the experimental observations in rat liver. A fall below this threshold for several hours is followed by hepatocytic injury (Decker et al, 1971). The administration of uridine is able to prevent and reverse liver cell damage induced by GalN (El-Mofty, et al, 1975).

The biochemical lesion described above results in the depression of uracil nucleotide-dependent biosynthesis of macromolecules, which include nucleic acids, glycoproteins, glycolipids in membranes, and glycogen. The depletion of these molecules leads to the injury of the organelles and the cytoplasmic membrane. Necrosis of the liver cells occurs eventually (Decker & Keppler, 1972).

1.4.2. Pathophysiology, Pathohistology and Pathobiochemistry of GalN Induced Liver Injury

Since UTP is the basic structural unit of ribonucleic acids (RNA), a depletion of UTP by GalN interference will inevitably impair the RNA synthesis. In addition, DNA synthesis may be reduced as well because uridylate is also a precursor of TTP. The disorder of RNA and protein synthetic machineries is also detrimental to DNA synthesis.

Both UDP-glucose and UDP-galactose are substrates of glycosyl transferase which is involved in the syntheses of glycoproteins and glycolipids. Depletion of these substrates is responsible for the low synthesis rate and the formation of the incomplete macromolecules.

Among the chain reactions of GalN intoxication is the impairment of the syntheses of the proteins including glycoproteins, such as coagulation factors, and the alpha$_1$-lipoproteins. The failure in lipoprotein synthesis causes the accumulation of fatty acids within hepatocytes. This is because the release of fatty acids from the liver cells

requires the formation of lipoproteins, and the synthesis of lipoproteins requires a carbohydrate moiety.

The rate of gluconeogenesis decreases after the induction of hepatitis (Monier & Wagle, 1971). One of the most drastically reduced enzymic activities is that of serum lecithin:cholesterol acyl transferase, which is a glycoprotein synthesized by the liver. Therefore, cholesterol ester levels diminish in plasma as a result of depressed hepatic synthesis. The same phenomenon has been proven in human parenchymal liver diseases (Phillips, 1960). Lysosomes in hepatocytes are injured and the activities of lysosomal enzymes in hepatic supernatants are recorded as being much increased. The lysosomal damage and the release of lysosomal enzymes activities due to plasma membrane fragility are partly responsible for the hepatic necrosis.

Light microscopic examination reveals scattered hepatocellular necrosis 6 hours after GalN administration. Kupffer cells appear enlarged. At 24 to 26 hours, foci of hepatocellular necrosis are disseminated throughout the lobules and hepatocytes are replaced by inflammatory infiltrates consisting mainly of segmented leucocytes, lymphocytes and plasma cells. The histologic changes are more severe 48 hours after GalN administration. Necrosis of extensive areas of hepatocytes, portal inflammation, and proliferation of bile ductules are very prominent (Medline et al, 1970).

The electron microscopic examination demonstrates hepatocellular injury as early as 2 hours after GalN injection

in rats. There appear to be an increasing number of autophagic vacuoles which contain the remnants of mitochondria, glycogen, and fibrillar and granular materials. Normal liver cells can no longer be observed 24 to 36 hours after the initial GalN injection. Cells appear ballooned and glycogen rosettes are almost completely disappeared. All organelles display various extents of injury, such as the dilatation of the rough endoplasmic reticulum and the irregular size and shape of the mitochondria. The nucleoli appear fragmented and dispersed. Almost all hepatocytes contain autophagic vacuoles and nonmembrane-bound fat droplets of various sizes (Medline et al, 1972).

Liver regeneration is observed 18 hours after GalN administration. Auto-radiography with ^{3}H-thymidine as a tracer has shown the increase in ^{3}H-labeling indices in different types of cells: Kupffer cells, bile duct epithelial cells, and periportal and centrolobular mesenchymal cells. (Lesch et al, 1970).

1.4.3. Suitability of GalN Model in FHF Research

Liver injury induced by large amount of exogenous GalN has many features in common with human fulminant hepatic failure. This model appears superior to the models induced by many other toxic agents, e.g., carbon tetrachloride, paracetamol, yellow phosphorous, anesthetics and dimethylnitrosamine.

To induce the animal models of fulminant hepatic failure, GalN can be administered via a number of routes. Injections

can be made intraperitoneally, intravenously and even subcutaneously. GalN given orally had no hepatotoxic effect in rats (Pickering et al, 1975).

The GalN-induced hepatic failure models have not only been produced in rats (Keppler & Decker, 1969; Pickering et al, 1975; El-Mofty et al, 1975; Chirito et al, 1977; Liehr et al, 1978; Chang et al, 1978; Chirito et al, 1979; Makowka et al, 1980; O'Neill et al, 1983; Ouchi et al, 1984), but also in mice (Chojkier et al, 1985), and rabbits (Blitzer et al, 1978; Ferenci et al, 1983; Schaffer et al, 1984; Pappas et al, 1984).

Chirito et al (1978, 1979) studied in detail the biochemical, hematological and histological changes in the GalN-induced FHF rat model. Their microscopic examination revealed extensive necrosis and complete dissociation of liver cells 48 hours after GalN intraperitoneal injection. Other organs like lung and kidney showed no morphological abnormalities. The levels of the liver-derived enzymes SGOT and LDH in the plasma increased rapidly and dramatically. These enzymes reached their peak 48 hours after GalN administration. This peak corresponded to the maximum liver damage. The progressively increased prothrombin time was accompanied by a bleeding diathesis in these animals. The counts of the erythrocytes, leukocytes and platelets were unchanged. The bilirubin level was increased by almost 10 fold. Blood glucose level fell below 50 mg/dl (normal value: 80-120 mg/dl). Cholesterol concentration in blood was raised to 3 times that of the control level. the above observations

show the similarities between this animal model and human FHF.

Other authors have reported that the serum biochemical tests and liver histology in GalN model reflect massive hepatic injury. They found that changes in plasma ammonia, amino acid concentrations, coagulation parameters and electroencephalogram were all similar to those found in human FHF (Blitzer et al, 1978).

According to the criteria set by Terblanche et al (1975), an appropriate model should meet the following requirements:

(1) Reversibility;

(2) Reproducibility;

(3) High mortality from liver failure;

(4) Large animal model;

(5) Minimal hazard to personnel.

GalN animal model has been proven to be highly reproducible provided that the strains of animals used are about the same age and weight (Decker & Keppler, 1972; Chang et al, 1978; Makowka et al, 1979). The liver damage is dose-related when GalN is given intraperitoneally or subcutaneouslly (Pickering et al, 1975).

A high mortality rate is obtained in this model. Owing to the very consistent dose-effect relationship the mortality can be controlled at a desired percentage by varying the amount of GalN injected per kg of body weight.

Jones' group of the National Institute of Health established a rabbit model of fulminant hepatic failure and the mortality of this model was comparable to human FHF (Blitzer et

al, 1978).

The reversibility of GalN animal models have been proved by many centers. Makowka and his coworkers (1979) studied the effects of intraperitoneal injection of dispersed single-cell suspensions of syngeneic hepatocytes, allogeneic hepatocytes, and bone marrow cells in GalN-FHF rats as an artificial liver support. They reported a highly significant improvement in survival rate of 60% and 70% in treated animals as compared to a control of 0%. When coated charcoal hemoperfusion was tested as a temporary liver support in rats with GalN-induced FHF, a significant improvement in survival rate was achieved: 70% in the treated group as opposed to only 30% in the controlled group (Chirito et al, 1977; Chang et al, 1978; Tabata & Chang, 1980). Another group in Japan obtained similar improvement in survival of their GalN rat model of hepatic failure. The homologous plasma cross-circulation has resulted in a significant increase of survival rate in a controled experiment (Ouchi et al, 1984).

The hazard to personnel is nearly zero.

Therefore, GalN-animal model has satisfactorily met with most of the criteria for an ideal animal model.

The only unfulfilled requirement is the size of the animal. GalN models are usually induced in small animals. However, the need for a large animal model is only conditional, depending upon the research objectives and the experimental design. For most investigations concerning the basic biochemical and pathological alterations, the small animal models of GalN-induced FHF will suffice.

1.5. MIDDLE MOLECULAR SUBSTANCES IN BLOOD AND BRAIN COMPARTMENTS IN FHF RATS

1.5.1. Middle Molecule Theories in FHF Research

The concept and the hypothesis of middle molecular substances being toxic metabolites in human diseases were first initiated in uremia research.

In 1972, Scribner's group proposed that middle molecules which are a group of molecules in the 500 to 5,000 molecular weight range, may be important neuropathological toxins in patients with chronic renal failure (Babb et al, 1972). Since then, extensive studies have been carried out at a large number of nephrology centers (Migone et al, 1975; Bergström & Fürst, 1976; Man et al, 1973). This group of middle molecules was first characterized by the swedish group of Fürst and Bergström in Karolinska Medical School as fraction 7 obtained from Sephadex G-15 gel permeation chromatography (Fürst et al, 1975). Fraction 7 was further separated into subpeaks 7a, 7b, 7c, 7d, 7e, 7f and 7g by ion-exchange resin chromatography. Based on laboratory and theoretical analysis and clinical trials with hemoperfusion, the possible role of middle molecules in hepatic coma was first postulated in 1972 (Chang 1972; Chang & Migchelsen, 1973). They were able to predict the existence of the "middle molecular toxic metabolites" in hepatic coma based on the clearance spectrum for larger molecules by the ACAC microcapsule artificial kidney. This hypothesis received further support when significant

improvement in the recovery of consciousness in grade 4 hepatic coma patients was achieved in hemodialysis using polyacrylonitrile membrane which has a high permeability for middle molecules (Opolon et al, 1976a; Opolon et al, 1976b). In pigs with surgically induced liver failure, hemodialysis using the high porosity polyacrylonitrile membrane resulted in slower deterioration of consciousness, less EEG impairment, and longer survival time when compared to hemodialysis using the lower porosity cuprophane membrane. The distinction between the two types of membranes is that polyacrylonitrile membrane has a higher molecular cut-off up to 5,000 daltons, therefore permits much more rapid transfer of larger molecules. On the other hand, cuprophane membrane only allows the permeation and transfer of small solutes such as salts, glucose, urea, uric acid, etc.. Williams' group in 1978 reported their experience in the treatment of fulminant hepatic failure by charcoal hemoperfusion and polyacrylonitrile hemodialysis (Silk & Williams, 1978). They reached the similar conclusion that the removal of middle molecules can lead to improvement of consciousness in hepatic coma. More recently, Leber et al also reported that total serum level of middle molecules was significantly increased in patients with hepatic failure due to acute viral hepatitis or intoxication by Amanita phalloides (Leber et al, 1980).

Presently, it is generally agreed that the rise in middle molecular metabolites is related to the severity of hepatic failure and the extent of hepatic coma; and that the removal of

some of these middle molecules may improve the recovery of consciousness and the survival time.

In regard to the source of these middle molecules, it was speculated that they could be derived from breakdown products of hepatocytes; hormones in abnormally high concentrations; or abnormal peptides accumulating secondary to the disturbances of hepatic metabolism (Shu & Chang, 1983). The molecular weight ranges of the middle molecules were proposed to be 1,000-1,500 (Leber et al, 1980); or 500-2,000 daltons (Chang & Migchelsen, 1973; Chang & Lister 1980; Shu & Chang, 1983).

The fact that both high porosity membrane hemodialysis and charcoal hemoperfusion can significantly improve the recovery of consciousness and survival time of FHF hepatic coma patients, while the cuprophane membrane hemodialysis can not, suggested that some of the responsible toxic substances may be within the middle molecular range. The difference in the clearance spectra between the two types of therapeutic measures was the molecules in the range of 500-5,000.

In spite of the above-mentioned facts and theories, little has been known in regard to the exact source, the chemical nature, the metabolic course, and the outcome of these middle molecules.

1.5.2. Methodologies in Middle Molecule Research

The first documented analytical study of uremic middle molecules was probably ascribed to the Polish group Lutz et al in 1971. They investigated the oligopeptides in blood plasma

and urine of healthy humans and of patients with nephrotic syndrome. They reported a statistically significant increase ($P<0.01$) in the concentrations of three oligopeptides, and the emergence of an additional oligopeptide in the blood plasma of uremic patients in comparison to the healthy control group (Lutz et al, 1972a; 1972b). Their method was based on liquid column chromatographies on Sephadex G-25 and QAE-Sephadex A-25 ion-exchange gels. They estimated that the molecular weights of these 4 acid oligopeptides were in the range of 500 to 1,200 dalton.

Dall'aglio et al, using various types of Sephadex gel chromatography, demonstrated the accumulation of middle molecules (MW<3,500) in sera, urine and dialysis fluid of uremic patients (Dall'aglio et al, 1972). In 1973, Dzurik et al isolated a peptidic toxin from the serum of chronic uremic patients by a series of liquid chromatographic procedures including Sephadex G-25 and DEAE-Sephadex A-25 (Dzurik et al, 1973).

Since then numerous laboratories have been researching for the separation and the isolation of uremic middle molecules. Among the various studies, the major techniques used can be classified into the following categories:

1) Liquid chromatographies: The most widely adopted technique is that of Fürst's group. This technique is mainly comprised of G-15 or G-25 gel size-exclusion, followed by ion-exchange chromatography using DEAE-Sephadex A-25 or QAE-Sephadex A-25 ion-exchangers (Fürst et al, 1976; Lutz et al,

1972; Dzurik et al, 1973; Chapman et al, 1980; Cueille et al, 1981).

2) Membrane filtration and dialysis: The membranes tested are: Amicon centriflo DM5 and UM05 (Dall'Aglio et al, 1972; Abiko et al, 1979); Amicon Diaflo XM50 (Leber et al, 1979); and Diaflo UM10, UM05 (Gutman et al 1980), etc..

3) Other techniques: There have been studies using thin-layer electrophoresis on Sephadex G-25 gel by Cichocki et al (1980); paper chromatography by Dzurik et al (1973); isotachophoresis by Zimmerman et al (1980); and thin-layer chromatography on silica by Leber et al (1979).

For analysis of hepatic failure middle molecules, the methodologies in principle are based upon the techniques used in uremic MM research.

The first analysis of the spectrum of molecules removed by coated charcoal hemoperfusion in liver failure patients suggested the probable involvement of middle molecules (Chang, 1972; Chang & Michelsen, 1973). Soon after this analysis, hemodialysis membrane prepared from polyacrylonitrile with high permeability to middle molecules began to be used in the artificial liver support systems. The observations of hepatic coma patients regaining their consciousness after high-permeability membrane hemodialysis gave further stimulation to the investigation of middle molecules. Faguer and his colleagues examined the existence of middle molecules in the plasma samples and brain tissue extracts of pigs with experimental acute hepatic encephalophathy induced by liver

ischemia (Faguer et al, 1980). Using the gel permeation chromatography on Bio-gel P4 and the UV and fluorometric detection, they demonstrated that two different peaks, corresponding to MW 1,000-4,000 were related to the course of liver failure. In 1980, The abnormally elevated middle molecular peak 7 and subpeak 7g were revealed in plasma, urine, liver and brain of experimental fulminant hepatic failure rats (Chang & Lister, 1980; Shi & Chang, 1982; Shu & Chang, 1983). The techniques used by these authors were a modification from Chapmann (1980), who employed the use of Sephadex G-15 and Sephadex DEAE A-25 gels for serial gel permeation and ion-exchange chromatographies of the body fluids and the tissue extract samples. Leber and his coworkers (1981) used the analytical procedures of serial gel filtration (Sephadex G-15) and thin-layer chromatographies to separate ninhydrin-stained substances from patients and rats with liver failure. They speculated that the molecular weights of these middle molecules were between 1,000-1,500 daltons. Recently, a Japanese group reported their results of middle molecule separation using membrane ultrafiltration with Amicon CF-50A membrane filters, and HPLC with size exclusion column (TSK G 2000 SW). They proposed that the abnormal HPLC peak b was a parameter of hepatic failure (Kawanishi et al, 1983; Nishiki, 1984).

Despite all the above investigations, there still remain a lot of questions to be solved in regard to the exact nature, molecular size, compartmental distribution, and the identity of the constituents of the hepatic failure related middle

molecules. More sophisticated techniques are required to accomplish the final goal of identifying these MM substances in fulminant hepatic failure.

1.6. ARTIFICIAL CELLS AND COATED CHARCOAL HEMOPERFUSION

The idea of artificial cells was demonstrated many years ago (Chang, 1957; Chang, 1964). Artificial cells are initially prepared by making use of some of the simpler properties of biological cells. Each artificial cell consists of an ultrathin semipermeable membrane of cellular dimensions enveloping or coating some biologically active materials. The enveloping semipermeable membrane separates the contents of the artificial cells from the external environment. The ultrathin membrane (about 20 nm in thickness) and the large surface/volume relationship (2.5 cm^2 in 10 ml of 20 μm-diameter artificial cells) allows for an extremely rapid equilibration of permeating molecules. The diffusion is 400 times faster than that in standard hemodialysis. Artificial cells can now be prepared to contain different materials, including enzymes, adsorbents, living cells and cell organelles (Chang, 1984). The medical applications of artificial cells have now included: artificial kidney, detoxification, immunosorbents, cancer therapy, red blood cell substitutes, enzyme replacement therapy, drug carriers, artificial liver support and enzyme immobilization (Sideman & Chang, 1980; Chang, 1984c).

1.6.1. Hemoperfusion using coated activated charcoal

Hemoperfusion, by definition, is the process whereby the anticoagulated blood passes through an extracorporeal column device and is reacted by the biomaterials (adsorbents, enzymes, etc) contained in the column device. The first experimental trial of hemoperfusion was performed in 1948 when Muirhead and Reid investigated the feasibility of direct blood contact with ion-exchange resins for the removal of ammonia (Muirhead & Reid, 1948). Severe adverse reactions such as muscle jerks, restlessness, and tachypnea in the experimental animals hampered the further trials. In 1964, Yatzidis first demonstrated that hemoperfusion using uncoated activated charcoal could remove various endogenous and exogenous toxins, including creatinine, uric acid, phenols, indoles, guanidines and organic acids (Yatzidis, 1964). He reported clinical improvement in patients with pericarditis, gastrointestinal symptoms and lethargy. In the subsequent years, Yatzidis and his colleagues reported more studies on the effects of uncoated-charcoal hemoperfusion for the treatment of acute drug intoxication and uremia. However, because of the severe adverse reactions due to charcoal hemoperfusion, the clinical suitability of this technique was seriously questioned by a number of investigators. Dunea and Kolff (1965) noted a 50% reduction in platelet concentration and significant hemolysis following charcoal hemoperfusion. Hagstam et al (1967) reported that embolization of fine charcoal particles and

profound thrombocytopenia were associated with hemoperfusion using uncoated-charcoal.

The use of activated charcoal microencapsulated within ultrathin polymer membranes is a major advancement in the application of hemoperfusion in medicine. Using the principles of artificial cells, Chang demonstrated that by coating the charcoal within a biocompatible membrane, platelet depletion and charcoal powder embolism can be effectively prevented (Chang, 1966; Chang, 1969; Chang, 1972a; 1972b). In 1970, the successful use of albumin-collodion-coated activated charcoal (ACAC) hemoperfusion in treatment of uremic patients was reported (Chang & Malave, 1970).

Since then, a growing number of researchers and clinicians have been actively involved in the development and application of hemoperfusion systems using the biomaterial-coated activated charcoal initially described by Chang. Clinical studies of coated-charcoal hemoperfusion system have been reported by the researchers in Canada (Chang, 1972; 1975; 1984; Chirito et al, 1977), the United Kingdom (Gazzard et al, 1974; Winchester et al, 1974; 1976; Williams, 1976), West Germany (Bandel, 1974; Leber et al, 1980), France (Opolon et al, 1978), Italy (Denti et al, 1975), Japan (Amano et al, 1978; Odaka et al, 1978), Norway (Thysell et al, 1975), China (Niu et al, 1980), and the United States (Andrade et al, 1971; Malchesky et al 1976; Rosenbaum, 1980). Activated charcoal has been coated with a variety of polymer materials including cellulose nitrate (collodion), cellulose acetate, acrylic hydrogel, methacrylic

hydrogel, polyhydroxy-ethyl-methacrylate, and biological macromolecules such as: gelatin, albumin, etc.. The clinical application of coated-charcoal hemoperfusion has now included acute drug intoxication, uremia, and fulminant hepatic failure (Chang, 1984). The improvements in the clinical application of coated-charcoal hemoperfusion have stimulated increasing industrial production of various ready-to-use commercial systems.

Since the development of the ACAC hemoperfusion system, Chang and co-workers have treated many uremic patients using this technique (Chang et al, 1984). In this way patients with severe symptoms of uremic neuropathy were maintained symptom-free with either hemoperfusion alone or hemodialysis combined with hemoperfusion. It was shown that 2 hours of hemoperfusion is as effective as 8 hours of hemodialysis with the systems used then. In patients treated by hemoperfusion, there were general improvements in nausea, vomiting, pruritus, peripheral neuropathy and the overall feeling of well-being.

In recent years, there has been remarkable progress in the design of various composite systems of combined hemoperfusion and hemofiltration, or hemoperfusion and hemodialysis (Chang, 1984). These systems not only retain the efficacy of the conventional hemofiltration and hemodialysis in removing sufficient amount of uremic small molecular metabolites, but also possess the added advantage of charcoal hemoperfusion in removing larger uremic molecules (500-1,300 dalton) which are believed to be responsible for some of the symptoms of uremic

neuropathy.

Coated charcoal hemoperfusion, has now been proven to be a very useful and effective technique in the treatment of various types of acute drug intoxication (Chang et al, 1973; Winchester et al, 1977; Rosenbaum, 1980; Chang, 1984b). It has been used successfully in treating patients with severe acute intoxication with glutethimide, methylprylone, methaqualone, barbiturates, salicylate, and paracetamol, etc.. Hemoperfusion with activated charcoal was proven more effective than hemodialysis in clearing the blood of those drugs most commonly associated with a life-threatening overdose. Furthermore, in such patients who were treated by charcoal hemoperfusion the coma time is markedly shortened and response to verbal command recovers very quickly (Rosenbaum, 1980).

1.6.2. Coated Charcoal Hemoperfusion in FHF Treatment

The use of coated charcoal hemoperfusion in the treatment of hepatic coma was first reported by Chang (1972). A 50-year-old woman suffering from grade 4 hepatic coma due to acute hepatitis was treated with intermittent hemoperfusion using albumin and collodion coated activated charcoal (ACAC). A temporary but complete recovery of consciousness was achieved during each of the three periods of hemoperfusion. No change of ammonia or bilirubin was recorded during this type of hemoperfusion. The improvement of consciousness in charcoal hemoperfusion was suggested to be associated with the removal of middle molecular and protein-bound substances (Chang, 1972;

1975). The first study of a large series of FHF patients that received coated-charcoal hemoperfusion was reported by Williams' group (Gazzard et al, 1974). The survival rate in this series was considerably better when compared to their previous consecutive 92 patients with grade 3 or 4 hepatic coma treated by conventional therapies over the past 4 years. However, further treatment of the subsequent 34 patients with the same hemoperfusion regime resulted in a very low survival rate. This was probably due to the problems of hemocompatibility related with the modified hemoperfusion columns in their later series of clinical trials. Thus, the overall survival in 71 patients of hepatic coma was only 23.9%, which did not show any statistical difference in comparison to 15.3% survial in the previous patients treated by conventional conservative therapy (Silk & Williams, 1978).

During the last decade, there have been an increasing number of reported clinical trials using coated charcoal hemoperfusion for the treatment of patients with hepatic coma (Abouna et al, 1974; Williams & Murray-Lyon, 1975; Blume et al, 1976; Gelfand et al, 1976; Amano et al, 1978; Chang, 1978; Bartels, 1978; Odaka et al, 1978; Silk & Williams, 1978; Winchester & Gelfand, 1978; Agishi et al, 1980). It is now generally agreed that charcoal hemoperfusion results in a higher recovery of consciousness than do conventional therapies. Corresponding to this consciousness improvement, the average survival time is also considerably lengthened. The survival rate in hemoperfusion treated hepatic coma (grade 4)

patients, however, is not significantly improved. Animal experiments have shown that hemoperfusion with the coated charcoal system could result in significant improvement in survival rates (70% compared to 30% in controls) when animals were treated in earlier grades of coma, but not in the later grades of coma (Chang et al, 1978; Tabata & Chang, 1980; Niu et al, 1980). Following this, clinical trials were carried out in patients with paracetamol induced FHF with grade 3 and 4 coma by Williams group (Gimson et al, 1982). They reported similar results showing that hemoperfusion started in grade 3 hepatic coma resulted in a significantly better survival rate (70% as compared to 30% in control) (Gimson et al, 1982).

1.6.3. Adsorption Mechanism of Activated Charcoal

The mechanisms of adsorption on activated charcoal are generally not specific. Extensive studies have shown that charcoal adsorption is an effective means for removing considerable quantities of organic compounds from aqueous solutions. Activated charcoal has been used for decades as an intestinal adsorbent for treating diarrhea. Due to its non-specific adsorption mechanism, charcoal removes a host of organic toxicants produced by intestinal bacteria. In industry, activated charcoal has been extensively used for cleansing waste-water. In recent years coated activated charcoal has been found to be very useful in the detoxification treatment of drug overdose and accidental intoxications in hemoperfusion(Chang, 1969; Agishi et al, 1979; Sideman & Chang, 1979).

Activated charcoal is a highly porous material prepared from the destructive distillation and carbonization of various organic materials, such as plants, sawdust, rice hulls, animal bones, carbon black, sugar, and petroleum, etc.. Charcoal is treated with high temperature, high pressure and acid washes, to increase its adsorption capacity. Such treatment yields activated charcoal.

The high adsorption capacity of activated charcoal is due to its high porosity and large surface area which can be as large as up to 1,000 m^2/g. Charcoal after activation has numerous pores throughout the whole particle. The pores can be classified into micropores (radius < 2 nm), macropores (radius

> 50 nm), and transitional pores (radius 2-50 nm). Adsorption of adsorbates in solution takes place in several steps. The solute diffuses (1) first through the liquid medium to reach the charcoal particle, then (2) through the coating membrane (if charcoal is coated), and (3) through the macropores, and finally (4) reach the surface of the micropores where most of adsorption process occurs (Giordano, 1980).

Charcoal adsorbs or holds the adsorbate with mainly physical interactions. The forces that govern adsorption at interfaces are dipole-dipole, dipole-polarizability and London dispersion forces which are responsible for attraction between adjacent atoms or molecules no matter how different their chemical natures are. London dispersion forces exist in all types of matter and are created by the interaction of fluctuating electronic dipoles with induced dipoles in adjacent atoms and molecules. Atoms and molecules are brought close together by London dispersion forces and yet kept a certain distance away due to the repulsive forces generated by the interpenetration of their electronic clouds (Giordano, 1980).

The adsorption rate of adsorbates onto activated charcoal in aqueous solution is found to be related to electrolyte concentration, pH, temperature, the adsorbate concentration, molecular size, the molecular configuration of the adsorbates, and the available surface area of the charcoal (Weber et al, 1963).

It was demonstrated that a decrease in pH probably results in a reduction of negative charges at the surface of some

charcoal preparations, thus favoring the adsorption of the negatively charged molecules. The general suppression of charges on the charcoal surface by alteration of ionic concentraion and pH of the solution, will likewise enhance the adsorption of non-ionic, or hydrophobic organic substances. As a general rule, nonpolar or hydrophobic solutes are better adsorbed from aqueous solution than polar or hydrophillic solutes (Denti & Walker, 1980). The optimal pH for adsorption of individual adsorbate depends on the ionic charge of the adsorbate. Adsorption on charcoal generally increases with decreasing temperature. Increasing the temperature of a system of physically adsorbed material will tend to desorb the adsorbate. This phenomenon has been used to allow cyclic reuse of the adsorbent. Adsorption has also a dependence on the initial concentraion of the adsorbate. Within a moderate concentration range, the more dilute the solution is, the more rapid the adsorption relative to the initial concentration is, meaning that a greater fraction of the total solute will be adsorbed in a given period of time. The size of the solute will affect the overall rate of adsorption. In general, the larger the molecule, the lower the rate at which it diffuses, hence the lower the rate it is adsorbed. It has been demonstrated that the adsorption rate declines with the increasing length of the aliphatic hydrocarbon chain and the increasing size of the solute. However, the adsorption for amphipathic molecules may display a different pattern. The expulsion of increasingly large hydrophobic components from

water permits an increasing number of water-water bonds to reform. Therefore, the adsorption rate of amphipathic molecules is generally increased with the addition of each CH_2 group to the hydrocarbon chain.

Apart from the characteristics of the adsorbate and its solution, the characteristics of charcoal per se also affects the adsorption rate through the variation of its particle size and concentration. It is obvious that the rate of adsorption on a solid surface will vary with the available surface area. In other words, for a constant mass of adsorbent, the adsorption rate will vary with the particle size of the adsorbent. The rate of adsorption should then increase with some function of the inverse of the diameter of the carbon particles. The smaller the particle size, the greater the surface area for a given mass of charcoal particles, and the greater the adsorbability. As far as the charcoal concentration is concerned, it appears quite clear that if given a fixed particle size, the adsorption rate varies linearly with the concentration of charcoal within certain range in the system (Weber et al, 1963).

Activated charcoal used in clinical and experimental medicine can be divided into many classes according to their physical shape and the method of manufacture. There are: (1) raw particulate; (2) extruded granular; (3) spherical; (4) membranous; (5) fibrous; (6) reticulated cylindrical; and other types of shapes manufactured to suit various purposes.

1.6.4. Tyrosinase Artificial Cells in Hemoperfusion

An effective means of selectively reducing the level of a given amino acid from blood was needed to study the cerebral response to such a reduction in the blood. Hemoperfusion using immobilized tyrosinase within artificial cells was thus chosen for this purpose.

Artificial cells containing enzymes, or multienzyme systems have been used in biomedical research. The enzymes are microencapsulated within a spherical ultrathin membrane, and prevented from direct contact with the external environment. Substrates which are permeable can equilibrate rapidly across the membrane to be acted on by the enzyme inside, and the product can freely difuse out. These enzymes within the cells can exert their action in vivo by a number of different routes of administration (Chang, 1980). These artificial cells can be implanted intramuscularly, subcutaneously, or intraperitoneally. They can also be administered intravenously, or into the gastrointestinal tract, or applied directly to the local lesions where certain enzyme is needed. In addition to all the above-mentioned applications, the microencapsulated enzymes can be used in the extracorporeal hemoperfusion system to act on substrates in blood circulation.

Artificial cells with immobilized tyrosinase have been tested and are feasible for the removal of tyrosine and free phenols in GalN-induced FHF rats (Shu & Chang, 1980; Shu &

Chang, 1981). Both in vitro and in vivo experiments have demonstrated that tyrosinase artificial cells used in hemoperfusion can significantly reduce blood levels of tyrosine and free phenols.

Tyrosinase is also known as polyphenol oxidase, monophenol and catechol oxidase. Tyrosinase is the only enzyme known which catalyzes the direct aerobic oxidation of monophenols, eg., tyrosine, phenol, p-cresol, 3,4-dimethyl phenol and 4-t-butyl phenol (Barman, 1969).

The present goal of using tyrosinase artificial cells in this thesis research was to examine the response of brain tyrosine concentration to the decrease of blood tyrosine concentration in hemoperfusion.

1.7. GOALS OF THIS THESIS PROJECT

I have three major goals for this Ph.D. research project which are given in the Sections 1.7.1. through 1.7.3..

1.7.1. Patterns of Amino Acid Distribution in The Plasma And Tissue Pools in GalN-FHF Rats

Extensive clinical and animal model studies have been carried out on disturbances of amino acid metabolism in cirrhosis or portal systemic encephalopathy (Iob et al, 1966; Fischer & Baldessarini, 1971; Fischer et al, 1974; Bollman et al, 1975; Smith & Rossi-Fanelli, 1978; Morgan et al, 1978; Ferenci & Wewalka, 1978; Sourkes, 1978; Tricklebank et al, 1978; James et al, 1979; Czygan et al, 1981; Weber & Reiser,

1982; Zanchim et al, 1983). Unfortunately, very little information is available in regard to the changes in amino acids in fulminant hepatic failure (FHF). Some studies have reported alterations in amino acid concentrations in the plasma (Rosen et al, 1977; Watanabe et al, 1982); in the brain (Mattson et al, 1970; Rigotti et al, 1982); in the plasma and brain (Record et al, 1975; 1976; Holmin et al, 1983; Denis et al, 1983); in the plasma and muscle (Roth et al, 1982). While these studies demonstrated some changes in amino acid metabolism, there is still not sufficient fundamental information of general amino acid disturbance in the body as a whole. Furthermore, the galactosamine induced fulminant hepatic failure rat model is being increasingly used as a model for drug induced fulminant hepatic failure. Very little basic information is available in regard to the amino acid disturbances in this animal model.

It is thus one of the goals of this thesis to look into the fundamental changes in amino acid changes among the major body fluids and tissues in the galactosamine induced fulminant hepatic failure rat model.

1.7.2. The Effects of Reduction of Plasma Amino Acid Levels on the Amino Acid Levels in the Central Nervous System

The findings of amino acid disturbance in liver failure have led to a great deal of research interest in maneuvering plasma amino acid concentration to effectively influence the amino acid concentrations in the central nervous system. Most

of the studies reported involve the intravenous infusion of amino acid solution enriched with branched chain amino acids (BCAA) in patients or animal models with portal systemic encephalopathy or cirrhosis (Fischer et al, 1974; 1976; 1978; Freund et al, 1979; Rossi-Fanelli et al, 1982; Eriksson et al, 1982; Marchesini et al, 1982). Some authors reported similar use of BCAA in infusional therapy or added to hemodialysis solutions in the treatment of fulminant hepatic failure (Fryden et al, 1982; Denis et al, 1983).

I have used another approach to study the effects of reduction in plasma amino acid concentrations on amino acid levels in the brain and CSF. Hemoperfusion using collodion coated activated charcoal was chosen as the means of reducing amino acid levels in plasma.

It is my objective to obtain the basic information on the effects of reducing plasma amino acid levels on the corresponding amino acid levels in the central nervous system. Coated charcoal hemoperfusion is used to remove a number of plasma amino acids. Artificial cells with immobilized tyrosinase were used in hemoperfusion to study the effect of removing a single plasma amino acid, tyrosine, on the level of tyrosine in the brain.

1.7.3. Preliminary Studies on the Disturbances in Middle Molecular Substances in Galactosamine Induced Fulminant Hepatic Failure Rats

As reviewed in Section 1.6., there remain many unsolved questions with regard to the existence and the nature of the

middle molecular substances (MM) in fulminant hepatic failure.

This research will attempt to obtain some basic information for a better understanding of the existence, the relative abundance, and the chemical characteristics of the middle molecular substances in rats with galactosamine induced fulminant hepatic failure. The changes of middle molecular substances in the plasma and brain in normal rats and FHF rats will be studied and compared. The effects of lowering plasma middle molecule levels by adsorbent hemoperfusion on the MM levels in the brain will also to be studied.

CHAPTER 2

MATERIALS AND METHODS

2.1. THE GALACTOSAMINE INDUCED FULMINANT HEPATIC FAILURE MODEL IN THE RAT

2.1.1. The Induction of GalN-FHF Model in Rats

Male Wistar rats were supplied by the Canadian Breeding Farm and Laboratories (Montreal, Canada). The rats used in this study were 70 $\pm$ 7 days old weighing 270-300 g. The animals were placed on a standard Purina Rat Chow diet with water ad libitum.

D(+)-galactosamine hydrochloride (2-Amino-2-deoxy-D-galactose; Chondrosamine) was obtained from Sigma Chemical Co. (St. Louis; MO). The galactosamine (GalN) solution was prepared just prior to use. The solution contained 90 mg GalN/ml sterile saline. The solution was adjusted to physiological pH 7.4 by dropwise addition of 2.5 N sodium hydroxide solution.

GalN was injected intraperitoneally at a dose of 90 mg/100 g body weight. GalN injection was always carried out at 2 to 4 P.M.. After GalN administration, the rats were maintained on the same Purina Rat Chow diet but the drinking water was replaced by 5 g/dl dextrose solution for the rats to drink ad libitum.

The maximum liver damage occurred between 24 hrs and 48 hrs after GalN administration. The mortality in rats with GalN-induced FHF is 70% - 80% (Chirito et al, 1977, 1979; Chang et al, 1978). Within 48 hrs of GalN administration, the rats rapidly developed hepatic coma of various grades. The hepatic

coma grading systems in animals are somewhat different from that in humans.

Grade 1 is defined as lethargic, and slow in movement.

Grade 2 characterizes drowsiness, retarded response to stimuli.

Grade 3 is defined as sleeping most of the time but arousable, responsive to painful stimuli and with occassional seizure.

In grade 4 coma, animals are completely comatose, not responsive to any stimuli, often with irregular respiratory rate.

All FHF rats used in this thesis research, unless otherwise specified, were in grade 3 coma when used for experiments or biochemical analysis.

2.1.2. Electron Microscopic Examination of the Hepatocytes

Normal rats and the rats in grade 3 hepatic coma 48 hours after GalN administration were used for the EM examination of the hepatocytes. Animals were fixed by systemic perfusion using 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The liver samples were diced into approximately 1 mm^3 cubes and immediately post-fixed in ice-cold 1% osmium tetroxide. After standard dehydration procedures, the tissue was finally embedded in Epon. Ultrathin sections were cut and post-stained with 4% uranyl acetate and Reynolds' lead citrate stain. Tissue sections were examined under Phillips 410 electron microscope.

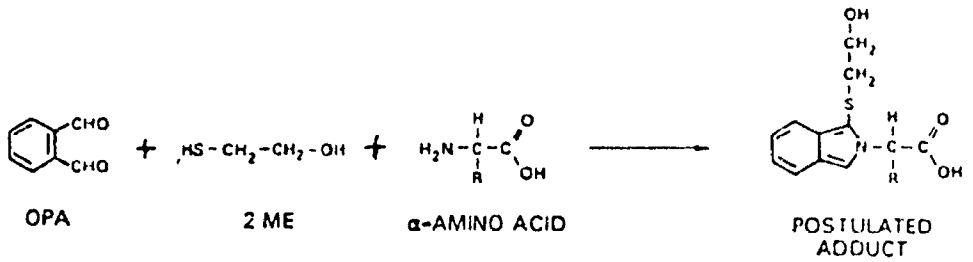
2.1.3. Measurement of Plasma Proteins

Blood samples were collected with atrial puncture from normal rats and rats with various grades of hepatic coma induced by GalN intraperitoneal injection. Fifty units of heparin was pre-added to each syringe for heparinizing approximately 5 ml blood. The heparinized blood was immediately centrifuged and the plasma samples were stored at -20°C. The plasma total protein and albumin analyses were carried out in the Clinical Biochemistry Laboratory at Royal Victoria Hospital with SMAC (Sequential Multiple Autoanalyzer Computerized). The protein analysis was usually carried out within 1-2 days after the collection of plasma samples.

2.2. AMINO ACID ANALYSIS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SYSTEM

2.2.1. General Principle

Primary amines react with ortho-phthaldialdehyde (OPA) and mercaptans to yield fluorogenic adducts that can be detected by fluorometers (Roth, 1971). The structure of the fluorescent derivative formed in the presence of o-phthaldialdehyde, a thiol, and a primary amine have been studied and illustrated by Simons and Johnson (1976; 1977). This derivatization reaction is illustrated as follows:



High performance liquid chromatography, especially with reverse phase columns, has been shown to have great efficiency, superb resolution, and rapid speed in the analysis of amino acids as their dansyl or phenylthiolhydantoin (PTH) derivatives (Engelhart et al, 1974; Haag et al, 1974; Bayer et al, 1976). Hill (1979) reported the successful HPLC determination of 20 common amino acids in biological fluids using OPA and 2-mercaptoethanol as derivatization reagents. This technique has greatly improved the speed, efficiency, sensitivity and specificity of chromatographic analysis of amino acids. Derivatized amino acids are eluted from the hydrophobic reverse phase ODS (C-18) column by an uphill gradient of organic solvent in the running eluent mixture. Amino acid peaks recorded on the chromatogram were therefore in the reverse order of hydrophobicity.

The HPLC amino acid analysis techniques used in this project were primarily adopted from Hill's method (Hill et al, 1979) with some modifications. As indicated in the folowing sections, some changes were made to meet the requirements of analyzing amino acids in plasma and tissue specimens.

2.2.2. Apparatus and Reagent Systems

A Waters high performance liquid chromatography (HPLC) system (Waters Associates Inc, Milford, Mass.) was used for the analysis of amino acids. This system is comprised of two solvent delivery pumps (Models M-45 and 6000A), a Waters Intelligent Sample Processor (WISP, Model 710B), a Data Module,

a System Controller (Model 720), and a Fluorescence Detector (Model 420-E). For fluorescence detection of derivatized primary amino acids, the excitation monochromator was set at 338 nm, and the emission was measured at 450 nm. Reverse phase micro-bondapak 5 μm ODS (C-18) columns (10 x 0.46 cm) were commercially packed by the Chromatographic Sciences (CSC Inc. Canada). A guard column (5 x 0.46 cm) packed with same material was always connected proximal to the main separation column.

Acetonitrile, methanol, tetrahydrofuran were all of HPLC grade (Fisher Scientific, USA). Mono- and di-sodium phosphate, boric acid, Tris, and potassium hydroxide were all of ACS grade (Fisher Scientific, USA). Buffers and solvents used in HPLC were all prepared with deionized water and were all filtered through 0.45 μm filters. Derivatization reagents were prepared in either of the following two ways depending upon the HPLC protocol:

(1) Reagent system 1:

Ortho-phthaldialdehyde (OPA) solution: Dissolve 100 mg OPA in methanol and add methanol to 10 ml mark in a volume metric flask.

Ethanethiol solution: Pipet 100 μl ethanethiol to a 10 ml volume metric flask, and then add methanol to a final volume of 10 ml.

Borate buffer: A 0.5 M solution was prepared and pH was adjusted to 10.5 by adding 3 M potassium hydroxide.

For derivatization, 1 ml sample or standards were added

with 0.5 ml borate buffer, 100 μl ethanethiol solution and 100 μl OPA solution. This mixture was then vortexed and filtered through 0.45 micron filter before injected to the column.

(2) Reagent system 2:

Borate solution: This solution was prepared the same way as in the reagent system 1.

OPA and 2-mercaptoethanol (2-ME) reagent mixture: Dissolve 50 mg OPA with 1 ml methanol in a 10 ml volume metric flask. Add 0.5 M borate buffer to 10 ml mark. Filter this solution through 0.45 micron nylon filter and then add 40 μl 2-mercaptoethanol. Store the reagent mixture in dark brown bottles at room temperature.

3) Amino acid standards:

The Amino Acid Standard H mixture solution was purchased from Pierce Chemical Company (Rockford, Ill.). Each 1 ml standard solution contained 1.25 μmoles and 2.50 μmoles each of the following: Ammonia as $(NH_4)_2SO_4$, L-lysine, L-histidine, L-arginine, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, glycine, L-alanine, L-valine, L-methionine, L-iso-leucine, L-leucine, L-tyrosine, and L-phenylalanine.

Since the Pierce Amino Acid Standard H mixture did not contain all amino acids that exist in biological samples, the standard solutions of the following individual amino acids were prepared: L-asparagine, L-glutamine, L-tryptophan, Taurine, L-ornithine, γ-amino-butyric acid (GABA), L-α-amino-butyric acid (Sigma Chemical Company, St. Louis, MO). These amino acids were dissolved in 20% methanol/water (V/V) solution and the

concentration was 5.0 mM. These individual amino acid standard solutions were then mixed together along with the Pierce Amino Acid Standard H solution and diluted with 20% methanol. The final concentration of each amino acid in standard solution was 100 M. This standard solution was prepared fresh every week. A further dilution of the standard solution to 25 μM was carried out immediately before the HPLC analysis.

Among these amino acid standards, several amino acids were not detected using the present technique. Proline is a secondary amine and does not react with OPA. Cystine also does not show fluorescence with the present derivatization method using OPA/2-ME or OPA/ethanethiol.

(4) Derivatization procedure:

One ml prefiltered sample or standards was mixed with 0.7 ml of reagent solution. The mixture solution is then vortexed and immediately injected into the Waters Intelligent Sample Processor (WISP). the elapsed time between reagent mixing and sample injection should always be less than 1 minute to prevent the degradation of the fluorescent amino acid adducts.

2.2.3. HPLC Elution Gradient

The hydrophobic chromatographic elution gradient was achieved by a two-pump, two-solvent system. Solvent A was a polar eluent and contained mainly an aqueous buffer solution. Solvent B was relatively a non-polar solution and contained a mixture of organic solvent and aqueous buffer. The elution was developed using an increasing percentage of solvent B as can be

seen from Table 2.2.3a. and 2.2.3b..

The aqueous buffer used was 15 mM phosphate solution. It was prepared in the following way:

(1) Dissolve 30.67 g Na_2HPO_4 (anhydrous) and 11.59 g NaH_2PO_4 in deionized water to make 1.0 l of 300 mM sodium phosphate stock solution;

(2) Dilute the stock solution by 20 times with deionized water to make the 15 mM phosphate working solution;

(3) Filter phosphate buffer through 0.45 micron cellulose acetate membranes (Milipore, USA) to ensure the high purity of the solvents for HPLC.

(4) Degas phosphate buffer solution before using. Degasing was done by connecting the vacuum suction to the side-arm of the flask containing the solution. Stirring the solution with a magnetic stirrer may accelerate the degassing process.

Solvent B which was used as a non-polar eluant was prepared by mixing an organic solvent (or solvents) with 15 mM phosphate buffer at a certain ratio (v:v). Solvent B was also filtered like solvent A but using a 0.45 micron nylon membrane (Milipore, USA) instead of a cellulose membrane. Degassing was also necessary before it could be used in HPLC.

Both solvents A and B were prepared fresh everyday and filtered and degassed as described above to ensure the best quality of the solutions.

HPLC GRADIENT TABLE

(METHOD 1)

Time (min)	Flow Rate (ml/min)	Pump A % Solvent A	Pump B % Solvent B	Gradient Curve
Initial	1.3	90	10	*
70	1.3	25	75	Linear
75	1.3	20	80	Linear
80	1.3	90	10	Linear
100 (Ready for next sample injection)	1.3	90	10	Linear

Table 2.2.3a. HPLC gradient table for method 1.

(1) Fluorogenic reagents: Use reagent system 1.

(2) Solvent A: 15 mM sodium phosphate buffer (pH 7.2).
Solvent B: Acetonitrile / 15 mM sodium phosphate buffer (55:45, V:V).

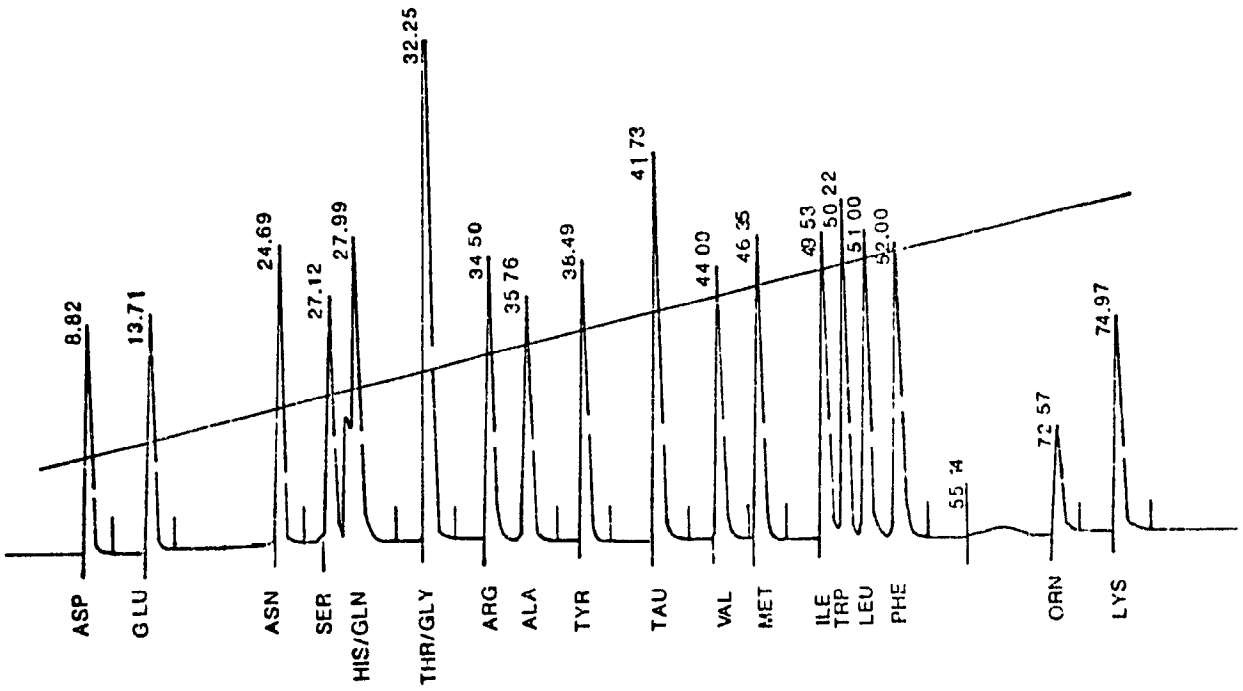


Fig. 2.2.3a. The standard chromatogram of 20 amino acids using reagent system 1 and gradient table 1. Using this technique, threonine and glycine are not separated from each other. Also, histidine is not resolved from glutamine. The numbers on the peaks indicate the elution time (min).

HPLC GRADIENT TABLE

(METHOD 2)

Time (min)	Flow Rate (ml/min)	Pump A % Solvent A	Pump B % Solvent B	Gradient Curve
Initial	1.3	100	0	*
5.0	1.3	99	1	Linear
55.0	1.3	20	80	Linear
60.0	1.3	10	90	Linear
65.0	1.3	10	90	Linear
70.0	1.3	100	0	Linear
90.0	1.3	100	0	Linear
(Ready for next sample injection)				

Table 2.2.3b. HPLC gradient table for method 2.

(1) Fluorogenic reagents: Use reagent system 2.

(2) Solvent A (2.0 l):
3 ml Tetrahydrofuran;
100 ml Acetonitrile;
1897 ml 15 mM sodium phosphate buffer (pH 7.2).

Solvent B (1.0 l):
30 ml Methanol;
570 ml Acetonitrile;
400 ml 15 mM sodium phosphate buffer (pH 7.2)

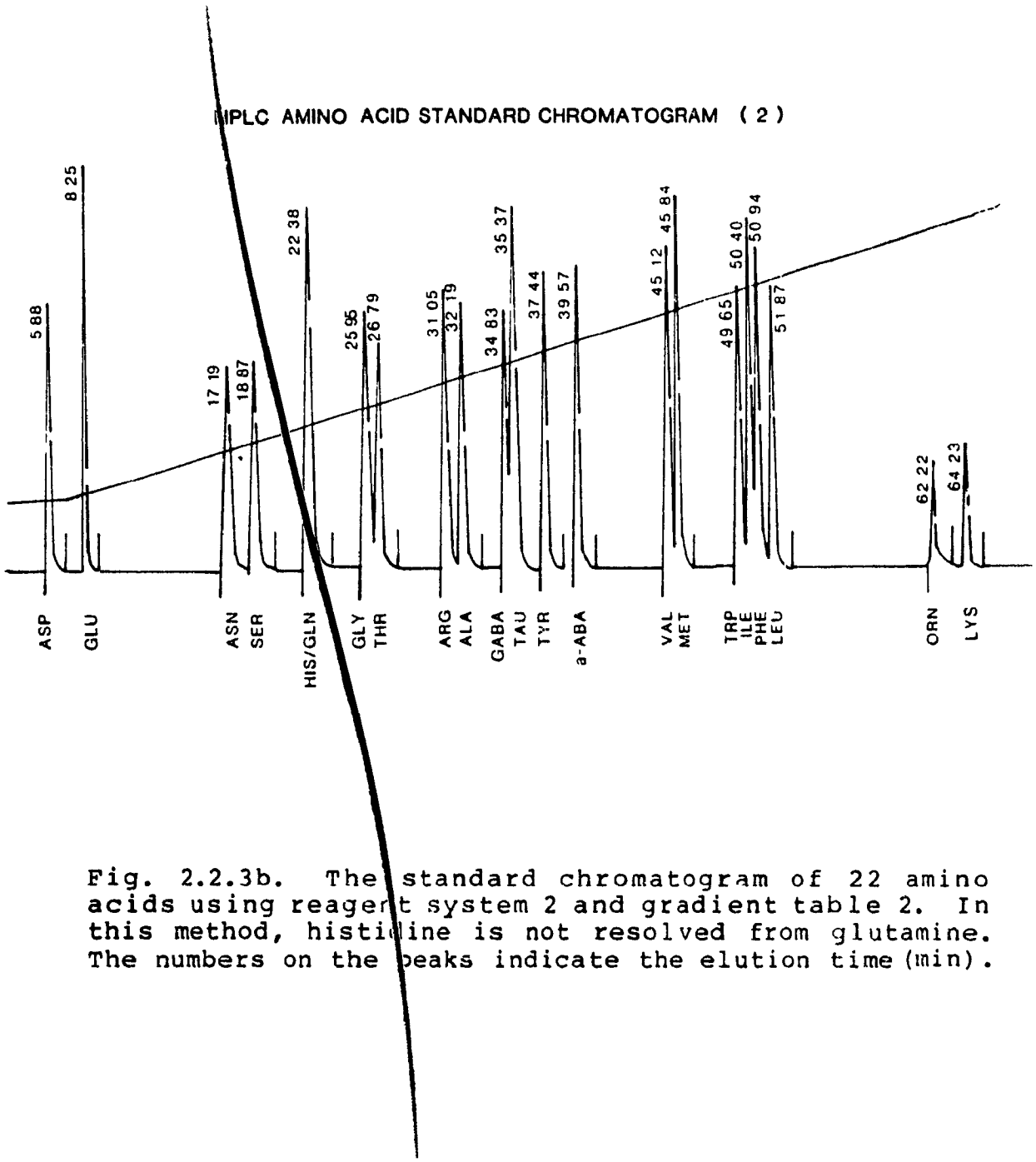


Fig. 2.2.3b. The standard chromatogram of 22 amino acids using reagent system 2 and gradient table 2. In this method, histidine is not resolved from glutamine. The numbers on the peaks indicate the elution time (min).

2.2.4. Plasma and Tissue Sample Preparation for HPLC Amino Acid Analysis

Rats were under light nembutal anesthesia for the sample-taking procedures. Cerebral spinal fluid samples were taken from the cisterna magna using a 27 G needle connected to a 250 µl glass syringe (for gas chromatography, by Hamilton Inc., Nevada, USA). By careful operation, approximately 150 µl of CSF could be drawn from each animal. The CSF samples were stored in 200 µl polyethylene micro-testtubes at -70°C. After CSF samples were taken, the animals were sacrificed by drawing blood directly from the right atria using a 10 ml syringe prefilled with 50 units of heparin. This heparinized blood sample was centrifuged at 4°C for 20 minutes and the plasma was then transferred to 1.0 ml Eppendorf centrifuge tubes for storage at -70°C. Immediately following the drawing of blood from the heart, the brain was carefully removed from the skull. The brain hemisphere (including cerebrum, diencephalon and mesencephalon) was dissected out, placed in glass vials and stored at -70°C The central left lobe (lobus sinister medialis) of the liver, the left kidney and the muscle from the right hind leg (caput mediale and caput lateral of muscles gastrocnemius) were excised and stored in glass vials at -70°C until HPLC analysis.

Just prior to HPLC analysis, the plasma samples were thawed. Deproteinization was done by adding 2.9 ml of methanol to 0.1 ml of plasma. After vortex mixing, the mixture was then centrifuged in an Eppendorf centrifuge for 4 minutes. The

clear supernatants were then filtered through a 0.45 micron nylon membrane with Millipore micro-filters. The filtrates were transferred to the reaction testtubes for fluorogenic derivatization for HPLC analysis.

The CSF samples were thawed and 50 μl was mixed with 5 volumes of methanol for dilution and deproteinization. Following vortexing and centrifugation, 200 μl supernatants were filtered in the same way as for plasma samples and 100 μl filtrates were used for fluorogenic derivatization.

All brain, liver, kidney, and muscle tissue specimens were thawed and weighed. The tissue specimens were homogenized in ice-cold saline (1:5, W:V) with a Polytron homogenizer (Brinkman Instruments, Ontario) at a speed setting of 6 for 1 minute. The homogenates were centrifuged at 15,000 g, 4^{0}C for 60 minutes in a refrigerated centrifuge. The supernatants were then deproteinized by adding 9 volumes of methanol. After vortexing and centrifugation, the supernatants were filtered through 0.45 micron nylon membrane filters. Aliquots of 200 μl filtrates were taken for fluorogenic derivatization for HPLC amino acid analysis.

For the analysis of amino acids in the brain regions, the brain was thawed and quickly dissected on ice into 4 major regions as reported by Rigotti et al (1982). These 4 brain regions are: (1) cerebrum (cortex and underlying white matter); (2) mid brain (diencephalon and mesencephalon); (3) cerebellum; and (4) brain stem (pons and medulla oblongata). These brain regions were weighed, and homogenized and centrifuged as

described above. The supernatants were treated the same way as that of other tissue extracts for HPLC amino acid analysis.

2.2.5. Analysis of Free and Total Tryptophan in Plasma and Tissues

The separation of free tryptophan from protein-bound tryptophan in plasma and tissue extracts was achieved using a Centrifree Micropartition System (Amicon Corp. Danvers, MA). This is a small compact centrifugal filter eqquipped with a cellulose acetate semipermeable membrane which has a nominal molecular cut-off at 30,000 daltons. In centrifugation, all protein molecules are retained while small molecules such as free amino acids are readily filtered through the membrane. Any protein-bound substances are also excluded along with the protein molecules. All small molecules in free form are recovered in the ultrafiltrate.

For analysis of free and total tryptophan, the plasma and tissue extract samples were equally divided into 2 portions. One portion (0.5 ml) of the sample was used for the total tryptophan measurement and was treated the same way as described in Section 2.2.4.. The other portion (0.5 ml) of the plasma or tissue extract sample was centrifuged using the Centrifree Micropartition System. Centrifugation was done in an refrigerated centrifuge at 2,000 g for 15 min. Approximately 0.4 ml ultrafiltrate was usually recovered from an initial load of 0.5 ml plasma or tissue extracts. This ultrafiltrate was then treated with serial procedures of

methanol dilution and fluorogenic derivatization.

The HPLC analysis of the free tryptophan was in the same way as that for other amino acids and total tryptophan which was described in Sections 2.2.2. and 2.2.4.

2.2.6. Manual Fluorometric Measurement of Tyrosine in Plasma, Liver and Brain

A fluorometric method was used for the measurements of plasma and brain tyrosines in this experiment. The method used here was adopted from the Manual of Fluorometric Clinical Procedures (GK Turners Associates, Palo Alto, California). The principle of this method involves tyrosine reacting with 1-nitroso-2-naphthol to produce a yellow compound with yellow-orange fluorescence. For plasma and tissue samples, a trichloroacetic acid filtrate was used and the fluorogenic reaction was carried out in the presence of nitric acid and sodium Nitrite. The reaction temperature was set at 60^{O}C using a water bath. Distilled water was then added. The unreacted 1-nitroso-2-naphthol was extracted using ethylene dichloride and discarded. The fluorescent tyrosine adduct which was left in the aqueous phase was then determined in a Turner Fluorometer (Model No. 111, GK Turner Associates, Palo Alto, California). Trichloroacetic acid and 1-nitroso-2-naphthol were purchased from Fisher Scientic Co. (Fairlawn, New Jersey). Sodium nitrite was obtained fom JT Baker Chemical Co. (Phillipsburg, New Jersey).

2.3. METHODS FOR HEMOPERFUSION STUDIES

2.3.1. In Vitro Adsorption of Amino Acids in Aqueous Solution by Coated Activated Charcoal

Two aqueous solutions of amino acids in 50 μM and 100 μM concentrations were prepared from Pierce Amino Acid Kit 22 (Pierce Chemical Co., Rockford, Illinois). Twenty primary amino acids normally present in the plasma were included in each of these solutions. Petroleum based spherical activated charcoal beads coated with cellulose nitrate(CAC) (Hemo-France, France) were used. Two grams of charcoal beads (CAC) were weighed and presoaked with distilled water and then placed into 20 ml of each of the 2 aqueous amino acid solutions in 50 ml flasks. The flasks were then gently oscillated in a Junior Orbit Shaker (Lab-line, USA) at room temperature. Samples taken before and after the 16-hour adsorption period were analyzed for amino acid concentrations by HPLC.

2.3.2. In Vitro Hemoperfusion Using Activated Charcoal for Amino Acid Removal

Galactosamine induced fulminant hepatic failure rats were used as blood donors. D-galactosamine was injected intraperitoneally into male Wistar rats at a dosage of 0.85-0.9 g per kilogram body weight. Forty-eight hours after galactosamine injection, blood was collected from the rats via cardiac puncture using 10 ml syringes preloaded with 50 I.U. heparin sodium USP (Allen & Hanburys, Toronto:Montreal). The heparinized blood from all FHF rats was pooled together and

then redivided into five 24 ml batches. Polypropylene hemoperfusion columns containing 1.5 g of collodion coated petroleum based charcoal beads (CAC) were used for this hemoperfusion study. Blood from the flasks were perfused at the flow rate of 1.0 ml/min through the charcoal cartridge. The flasks containing FHF rat blood were constantly oscillated using a Junior Orbit Shaker. Samples were taken from the inlet and the outlet of each column at 15 or 30-minute intervals. Clearance was calculated according to the equation:

$$\text{Clearance} = \frac{AA_{in} - AA_{out}}{AA_{in}} \times \text{Flow rate (ml/min)}$$

Where AAin is the amino acid concentration in the inlet plasma flowing into the hemoperfusion shunt, and AAout is the amino acid concentration in the outlet plasma from the shunt.

2.3.3. In Vivo Hemoperfusion Using Coated Activated Charcoal

Male Wistar rats weighing 265-310 g were injected intraperitoneally with D(+)-galactosamine hydrochloride (Sigma, USA). Forty-eight hours after galactosamine administration, those rats in grade 3 hepatic coma were randomized into 2 groups, a hemoperfusion treated group and a control group. All animals were anesthetized by intraperitoneal injection of Somnotal (Sodium pentobarbital, by M.T.C. Pharmaceuticals, Hamilton, Canada) at a dosage of 2 mg/100 g body weight. This dosage was only one quarter of that in normal animals. To heparinize the animals and the extracorporeal hemoperfusion circuit, heparin was given intravenously and also added to the

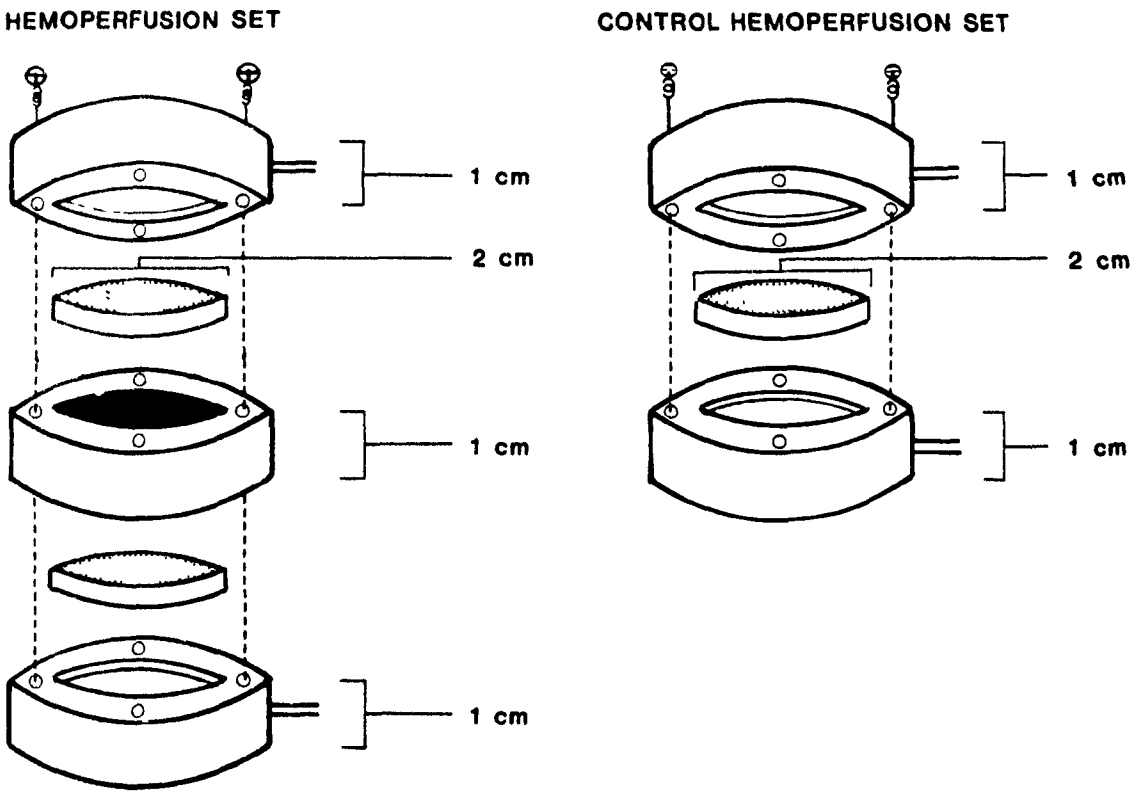


Fig. 2.3.3. The schematic diagram of the hemoperfusion columns. The columns are made of polypropylene. The column on the left side is used for charcoal and tyrosinase artificial cell hemoperfusions. This column is composed of a central chamber, two stainless steel meshes, and two covering pieces on the top and the bottom. Blood enters the inlet from the bottom covering piece, flows through the central chamber, and leaves the column from the top covering piece. The column on the right side is used for the control hemoperfusions. The central chamber piece is removed.

priming solution of the cartridge at 10 USP units/100 g body weight. Using a Minipulse pump, arterial blood was pumped through the hemoperfusion column which contained CAC charcoal beads and back to the body via the cannula in the femoral vein. PE-10 (I.D. 0.28mm) and PE-50 (I.D. 0.58 mm) polyethylene tubings (Clay Adams, Parsippany, New Jersey), were used as catheters for cannulating the blood vessles.

The set up of in vivo hemoperfusion shunt was similar to that of in vitro hemoperfusion. Polypropylene columns containing 1.5 g collodion coated petroleum based bead-type activated charcoal (CAC) (Hemo-France, France) were used for hemoperfusion treated animals (Fig. 2.3.3.). For control hemoperfusion, the columns contained no charcoal beads but only priming solution (Fig. 2.3.3.). The size of the control hemoperfusion columns was also reduced by removing the middle piece of the chamber, but leaving the top and the bottom pieces in position. Since the top and bottom pieces of the chamber did not constitute any inner space, the remaining parts of the control hemoperfusion chamber composed only a negligible inner space volume.

The flow rate for hemoperfusion in both test and control animals was maintained at 0.6 ml/min using a Minipulse peristaltic pump (Gilson, France).

2.3.4. Tyrosinase Artificial Cell Hemoperfusion

Tyrosinase (E.C.1.14.18.1) was obtained from Sigma Chemical Co. (St. Louis). When tested with L-tyrosine as

substrate, its activity is 2,230 units/mg enzyme. One unit is equivalent to an increase in 0.001 UV absorbance unit (280 nm), at pH 6.5 at 25°C.

Tyrosinase artificial cells were prepared with collodion cellulose membrane using the method of interfacial coacervation (Chang, 1972). A 10% hemoglobin (Worthington Biochem Corp, Freehold, New Jersey) solution was first prepared and the pH of this solution was adjusted to 7.4 with addition of Tris (hydroxymethyl) amino methane. This solution was then filtered through Whatman No.42 filter paper to remove any residual particles. Tyrosinase was dissolved in this 10% hemoglobin solution to a concentration of 1 mg (2,230 units)/ml. This enzyme-hemoglobin solution was then ready to be microencapsulated within the collodion membrane.

A collodion (cellulose nitrate) solution was prepared with the updated technique (Yu & Chang, 1980) to consist of 4.0 g cellulose nitrate (Fisher Scientific Co., Montreal) in a solution of 17.5% (volume) alcohol and 82.5% (volume) ethyl ether.

To a 150 ml glass beaker in an ice bath the following were added: 4.0 ml hemoglobin solution containing 4 mg tyrosinase, and 40 ml of water-saturated ethyl ether. The mixture was immediately emulsified for 5 seconds using a Jumbo magnetic stirrer (Fisher Scientific Co., Montreal) at a speed setting of 3. Without stopping the stirring, 40 ml of collodion solution were added and the reaction mixture was stirred for a further 1 minute at the same speed. The stirring process created

numerous microdroplets containing the enzyme solution. The beaker was then covered with aluminum foil and placed in a refrigerator at 4°C for 45 minutes to allow the gradual precipitation of cellulose ester at the interface between the ether solution and the suspending microdroplets. The supernatant was removed and 50 ml of n-butyl benzoate (Eastman Kodak Co., Rochester, New York) containing 1% (V/V) Span-85 (Atlas Chemical Industries, Brantford, Ontario) were added and the mixture was stirred for 30 seconds at speed 5. The suspension was then left at 4°C for 30 minutes to allow ether to evaporate and the outer surface of the microdroplets to set. The microcapsules (artificial cells) thus formed were repeatedly washed with Tween-20 solutions (Atlas Chemical Industries, Brantford, Ontario) and rinsed with deionized water. Normal saline was used to wash away any residual Tween-20 and other chemicals. Finally, the microcapsules were resuspended in normal saline or lactated Ringer's solution for the hemoperfusion experiment.

For control hemoperfusion, the collodion microcapsules were prepared containing only hemoglobin solution but no enzyme. The other procedures for preparing control microcapsules were identical to the enzyme microcapsules.

The hemoperfusion columns and the extracorporeal blood circuit were all set up the same way as that of charcoal hemoperfusion which has been described in section 3.2.3. of this thesis. Control hemoperfusion was performed in a similar way to tyrosinase artificial cell hemoperfusion except for the

columns that contained only hemoglobin microcapsules without tyrosinase.

The animals used for tyrosinase artificial cell hemoperfusion were male Wistar rats with GalN-induced FHF. The procedures for preparing this animal model have already been described (Section 2.2.1. of this thesis). The animals were randomly divided into two groups for either tyrosinase hemoperfusion or control hemoperfusion (without tyrosinase) experiments. The hemoperfusion time was 2 hours. The flow rate was maintained at 0.6 ml/min using a Minipulse peristaltic pump (Gilson, France). Blood samples were taken before, in the middle of, and at the end of the 2-hr hemoperfusion for tyrosine measurements.

2.3.5. Hemoperfusion for Middle Molecule Removal

The general procedures of animal preparation for middle molecule studies were similar to that of amino acid studies (Section 2.2.1.). Briefly, fulminant hepatic failure was induced in male Wistar rats by the intraperitoneal injection of galactosamine solution at a dosage of 0.85-1.0 g/kg body weight. Forty-eight hours after the injection, rats in grade III hepatic coma were used for the baseline middle molecule studies in comparison with normals, as well as for the charcoal hemoperfusion experiments in comparison with controls.

The animals were under light nembutal anesthesia before they were sacrificed for sampling blood and brain in the baseline middle molecule studies. Those rats in hemoperfusion

studies, were also anesthetized by light nembutal (1/3 of normal dosage) and cannulated at the femoral arteries and veins to gain blood access for hemoperfusion. Blood from the cannulated femoral artery is pumped through a hemoperfusion column containing collodion-coated activated charcoal beads (CAC), then returned back to the animal through the venous cannula. Heparinization was achieved at a total dosage of 10 iu/100 g body weight. Blood specimens were taken before and after 1 hour hemoperfusion. Brain cortex samples were taken immediately after hemoperfusion. Control animals were treated the same way except they were not hemoperfused. Blood and brain specimens from both treated and control animals were prepared in the same way for chromatographic analysis of middle molecular substances.

2.4. MEASUREMENTS OF MIDDLE MOLECULES IN BLOOD AND BRAIN

2.4.1. Sephadex G-15 Gel Permeation Chromatography

Heparinized blood was first centrifuged. Plasma was filtered through CF-50A membrane cones (retentivity: 50,000 daltons, from Amicon Company) by centrifugation for 20 min at 1,000 g. The ultrafiltrate was applied to Sephadex G-15 gel permeation column (size: 75.0 x 0.4cm) and eluted with 0.01 M TRIS buffer (pH 8.6 preadjusted with 0.02 N HCl). The UV absorbance of eluate was monitored at 254 nm or 206 nm with a Pharmacia UV-1 detector. The recorded peaks were named numerically in the reverse order of elution volume. Thus the

first peak was named peak 9 and the last, peak 1. Peak 9 which is comprised of macromolecular substances, eg., plasma proteins, etc., was eluted at the void volume of the gel column. Peak 7, which contains molecules in the middle molecular weight range (500-1500 daltons), is eluted after peak 9 and collected with a Pharmacia fraction collector. Peaks from 5 to 1 contained only small solutes and were not collected.

Brain specimens were homogenized in 0.01 M TRIS buffer (1:5, W:V), with an ice bath. Homogenization was done with a Polytron Homogenizer at a speed setting at 6 for 1 min. The homogenates were centrifuged at 10,000 g in a refrigerated International Centrifuge for 20 min. The supernatants were then filtered through CF-50A membrane cone filters. The ultrafiltrates were collected and applied to G-15 columns for liquid chromatographic analysis of middle molecular substances using the same procedures as for plasma samples.

G-15 Sephadex column was calibrated with molecular weight standards (Fig. 2.4.1.). Blue dextran was used to determine the void volume of the column. Vit.B12 (MW 1,355) was an indicator of the middle molecular peak(s). Creatinine and uric acid (MW 113 and 168, respectively) are indicative of the bed volume of the column.

2.4.2. DEAE-Sephadex Anion-Exchange Chromatography

Fractions of peak 7 eluates from G-15 gel permeation were subjected to further separation, ie., DEAE-Sephadex anion exchange

SEPHADEX G-15 GEL PERMEATION CHROMATOGRAPHY

OF MOLECULAR WEIGHT CALIBRATION

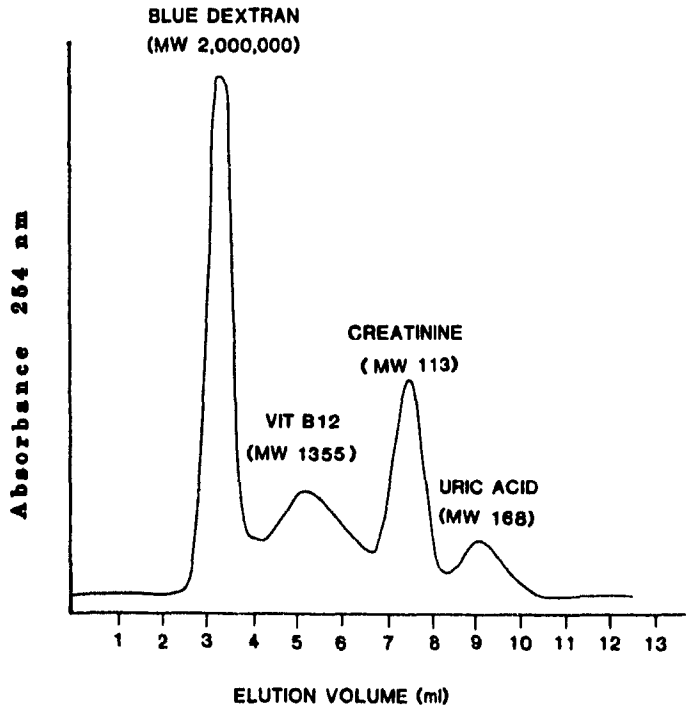


Fig 2.4.1. The chromatogram of the molecular weight standards using Sephadex G-15 gel column chromatagraphy.
Column size: 75.0 X 0.4 cm glass column.
Solvent: 0.01 M TRIS buffer (pH 8.6, adjusted with 0.02 N HCl).
Flow rate: 12.5 ml/hr.
Monitor: 254 nm using a Pharmacia UV-1 detector.

chromatography. Glass columns (size: 30.0 x 0.4 cm) were packed in pairs, designated as sample and reference columns, with pre-swollen DEAE-Sephadex gels in 0.01 M TRIS buffer (pH 8.6, adjusted with 0.02 N HCl). A Pharmacia gradient mixer was used to establish an increasing ionic gradient of sodium chloride from 0 to 1 M in 0.01 M TRIS buffer. The gradient elution was initiated as soon as the sample of peak 7 fraction was loaded onto the sample column. Both sample and reference columns were concurrently eluted with the same gradient buffer and both eluates were monitored so that the baseline shift of UV absorbance due to the solvent gradient would be compensated for. The UV monitor was set at 1% the sensitivity that of G-15 gel permeation due to the much diluted nature of the solutes in eluates. In gel permeation, the UV sensitivity was set at 2.0 absorbance unit/full scale, and in anion-exchange chromatography it was set at 0.02 unit/full scale. UV absorbance was monitored at 254 nm using a Pharmacia UV-1 Monitor. The subfractions of Peak 7 were named alphabetically as subpeaks 7a, 7b, 7c, 7d, 7e, 7f and 7g in the reverse order of the elution time.

The middle molecule levels were quantified in Peak Height Units (PHU) per ml of plasma or g of wet tissue. One PHU is 0.01 absorbance unit measured at 254 nm.

2.4.3. Detection of Peptidic Substances in Middle Molecular Fractions from Liquid Chromatographies.

The ion-exchange chromatographic subfractions of peak 7

were tested for the existence of peptidic substances using Bio-Rad Protein Assay reagents. The standard protein solutions were prepared using the Total Protein Calibrator (American Monitor Corporation) in the concentration range from 2 to 100 μg/ml. The TRIS buffer, same as that used in the gel permeation and anion-exchange chromatographies, was used for diluting the standards. The micro-assay techniques were adopted to test the minute amount of peptidic substances. Each subfraction from ion-exchange chromatography was 1.0 ml in size and was added directly with 0.2 ml Bio-Rad Protein Assay reagent. The use of the Eppendorf multiple dispenser for adding the reagents insured the relative accuracy of the assay. All 30 fraction tubes were gently vortexed to mix the reagent with the eluates and were stood at room temperature for 10 min. The protein assay reagent contains Coomassie Brilliant Blue G-250 which shifts its absorbance maximum from 465 nm to 595 nm when binding to proteins or peptides. The protein standard solutions were first reacted and measured at a Beckman T720 spectrophotometer to plot a standard curve with absorbance unit values against the concentration levels. The absorbance of sample solutions were then measured and recorded. The individual sample values were read off the standard curve, and the concentrations of peptidic substances in all 30 fractions of ion-exchange chromatographies were plotted on a separate graph chart.

2.4.4. SDS-Polyacrylamide Gel Electrophoretic Detection of Peptides in FHF Plasma

In 1971, Swank and Munkres first described the molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (Swank and Monkres, 1971). The techniques in my experiment were mostly adopted from their paper with some modifications. A polypeptide molecular weight (PMW) Electrophoresis Calibration Kit was purchased from Pharmacia Fine Chemicals. The kit consists of vials containing a lyophilized mixture of well characterized standards obtained from the partial cleavage of sperm whale myoglobin with cyanogen bromide. The molecular weights of the peptide standards ranged from 1,695 to 17,201. The standards were dissolved in a sample buffer which was prepared as follows: Adjust a solution of 0.01 M H_3PO_4, 8 M urea to pH 6.8 with TRIS. Add sodium dodecyl sulfate and β-mercaptoethanol to a final concentration of 2.5% and 5% respectively. A tracking dye, bromophenol blue, was added to a final concentration of 0.02%. The electrophoretic running buffer was prepared with 0.1 M phosphoric acid which was adjusted to pH 6.8 with TRIS, and SDS was added to 1%. The gels were cast into 2 layers: the separating gel (%T=13.8, %C=9.1) beneath the stacking gel (%T=6.9, %C=9.1). The separating gel, which is also called the running gel, was prepared with the running buffer and 8 M urea. The stacking gel was prepared in a similar way except the concentrations of the buffer and urea were only half that of the running gel.

The inclusion of the stacking gel was indispensable here since it resulted in sharper and better resolved bands.

The plasma samples were prepared as follows: Fifty μl of plasma from either normal or FHF rats were diluted with 150 μl sample buffer, which was the same buffer used for the reconstitution of the standards. This buffer denatures proteins and helps to stabilize the peptides. The diluted samples were then vortexed and centrifuged to sediment the protein precipitates. Supernatants were transfered to 0.5 ml size Eppendorf centrifugal tubes. To each 50 μl supernatant, the following reagents were added: 10 μl glycerol, 4 μl β-mercaptoethanol and 8 μl 0.1% bromophenol blue. This mixture was gently vortexed and then 50 μl from each mixture was carefully loaded into the sample wells in the stacking gel. Electrophroresis was carried out using a Bio-Rad vertical slab electrophoresis cell (Model 220) at 80-100 volts for 12-14 hours and was terminated when the tracking dye bromophenol blue reached 1 cm from the bottom. The slab gels were then subjected to a 2-step staining procedures: 1) Staining with 0.02% Coomassie Brilliant Blue R250 using the methodology by the Pharmacia Fine Chemicals (1984); 2) Silver staining using the techniques provided by the Bio-Rad Laboratories(Canada) Ltd (Bio-Rad Bulletin No. 1089, 1982).

2.4.5. Radio-Immunoassay of Beta-Endorphin in Plasma and Brain Samples from Normal and FHF Rats

Radio-immunoassay was performed to investigate whether β-

endorphin was involved in the FHF related middle molecules. This study was carried out in the Montreal Clinical Research Institute and the reagents and the equipments were generously supplied by Dr. John Chang. This experiment included 6 normal rats and 6 rats in grade III hepatic coma. Both normal and hepatic coma rats were decapitated using a guillotine without anesthesia.

Both the blood samples from the normal and the FHF rats were prepared the same way for β-endorphin RIA. Blood was centrifuged for 15 min at 4°C, and was transfered to Eppendorf centrifugal tubes (1.5 ml size). To each 0.5 ml plasma 50 μl of 10 μM α-phenylmethylsulfonyl fluoride (PMSF) was added to protect β-endorphin from the attack of the proteolytic enzymes in plasma.

The following procedures were followed to extract β-endorphin from plasma samples:

(1) Distilled water was added to each 0.4 ml plasma sample, to make a final volume of 2.0 ml;

(2) Add 50 mg silicic acid, shake for 1h at 4°C;

(3) Centrifuge at 10,000 g at 4°C, discard supernatant;

(4) Add 2 ml distilled water, votex, and repeat step 3;

(5) Add 0.5 ml 3 N HCl, vortex, and repeat step 3;

(6) Add 1 ml 30% acetone/70% HCl, vortex, centrifuge, use 0.1 ml supernatant for each duplicate assay tube for RIA.

The brain samples (excluding brain stem and pituitary) were carefully dissected from the skulls immediately after the decapitation of the rats and were stored at -20°C until the day

for RIA. All brains were thawed on ice before RIA, weighed and homogenized in 10 ml 0.2 N HCl with an ice bath using a Polytron Homogenizer at speed setting 6 for 30 sec. Homogenates were centrifuged at 10,000 g for 1 hour in a refrigerated Beckman centrifuge. Supernatant was transfered and diluted to 1/10 with 9 volumes of RIA buffer. One hundred µl of each sample were used for RIA. One liter of RIA buffer was composed of 0.05 M phosphate (pH 7.4), 10 ml 25% human albumin, and 10 mM EDTA.

The double antibody precipitation technique was used for β-endorphin RIA and the procedures were as follows:

1) Set up, in duplicates, a series of β-endorphin standards from 0 to 1,000 pg/tube;

2) To each 100 µl standard or unknown sample, add 100 µl β-endorphin antibody, and 100 µl RIA buffer, gently vortex to mix;

3) Incubate at $4^{o}C$ for 48 hours;

4) Add 100 µl ^{125}I labeled β-endorphin, incubate at room temperature for 3 to 4 hours;

5) Add 100 µl of second second antibody to each tube, gently vortex;

6) Centrifuge at $4^{o}C$, discard the supernatant;

7) Read CPM of the precipitates, in a gamma scintillation counter.

The standard curve was plotted on a semi-logarithmic graph with percentage of antibody bound ^{125}I-β-endorphin (CPM) over total counts (CPM) as the ordinate, and the standard

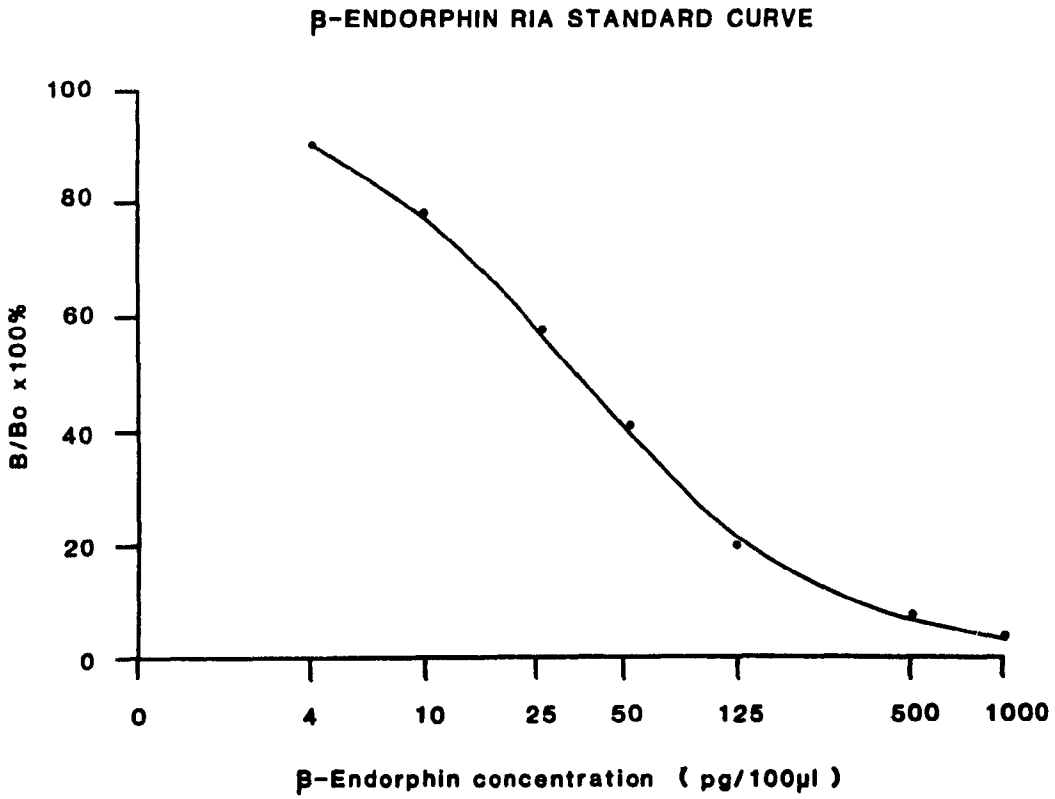


Fig. 2.4.5. Beta-endorphin radio-immunoassay standard curve. This is set up using double antibody precipitation technique.

concentration as the abscissa (Fig. 2.4.5.). The concentration of the unknown samples were read off the standard curve. The recovery rate of the extraction was calculated with re-extraction of the standards which were added to the pre-extracted plasma.

2.4.6. Radio-Immunoassay of Substance P in FHF Rat Plasma

The substance P RIA kit was purchased from Immuno-Nuclear Corporation (Minnesota, USA). This RIA employs simultaneous addition of sample, rabbit anti-body and ^{125}-I substance P, followed by an overnight incubation at 4^{o}C. The phase separation is accomplished by the addition of an equal volume of saturated ammonium sulfate in the presence of carrier gamma-globulin, ie., the second antibody. The assay tubes were then counted in a gamma scintillation counter for the ^{125}I CPM in the Substance P-antibody complex precipitates.

Substance P standards were serially diluted using bovine serum albumin-peptone buffer to give concentrations of 0, 19.5, 39, 78, 156, 312, 625, and 1250 pg/ml. The standard curve was in a sigmoid shape (Fig. 2.4.6.).

Plasma samples taken from normal and FHF rats were prepared in the following procedures for the extraction of substance P:

1) To 1.0 ml of heparinized plasma, add 2.0 ml cold acetone. Vortex and centrifuge to extract, then transfer the supernatants to another polypropylene tube;

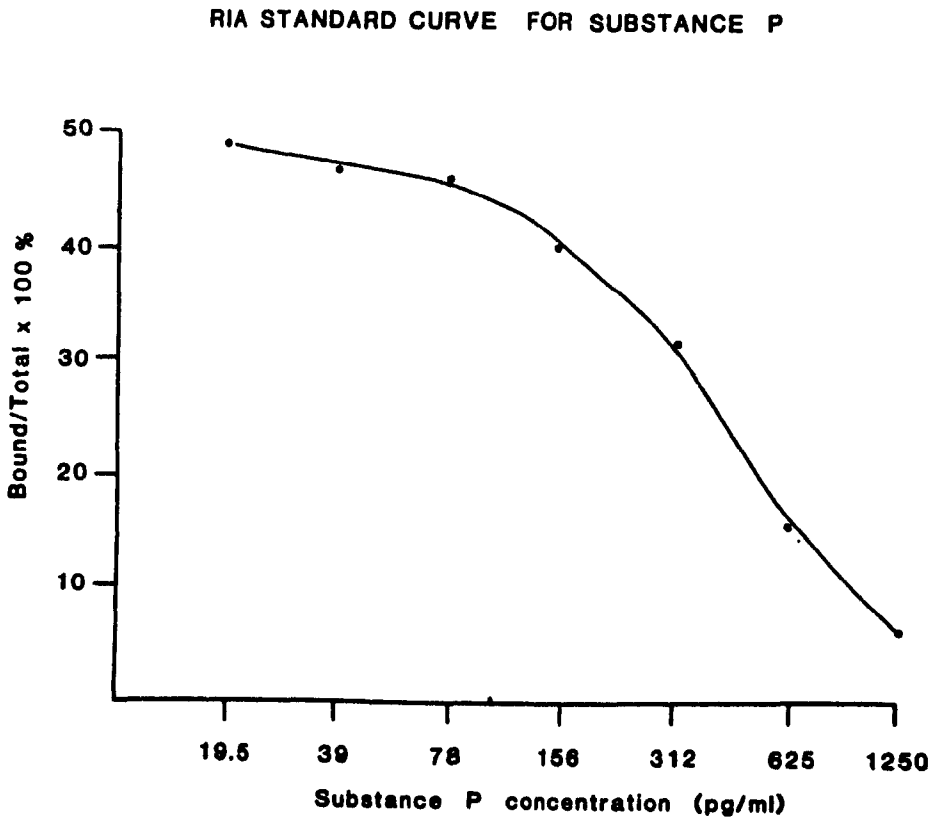


Fig. 2.4.6. Substance P radio-immunoassay standard curve. This is established using a RIA Kit from Immuno-Nuclear Corporation (Minnesota, USA).

2) Defat the supernatants by adding 4.0 ml petroleum ether to each tube, mix and centrifuge, aspirate and discard the upper layer of petroleum ether;

3) Dry the lower aqueous layer at 37°C in a water bath with a clean air flow which was created by a vacuum suction;

4) Reconstitute the dried extracts with 0.5 ml BSA-peptone buffer and vortex to dissolve;

5) Incubate for 10 min at 37°C and mix well for RIA.

The above extraction procedure has a reported recovery of 74-119% (Immuno-nuclear Corporation, 1984).

The percentage of bound over total ^{125}I-substance P was calculated using the following equation:

$$\frac{\text{Bound}}{\text{total}} \times 100\% = \frac{\text{CPM of STD or sample}}{\text{CPM of Total count tube}} \times 100\%$$

A standard curve was constructed on a semi-logarithmic graph paper by plotting the percent bound (B/T%) on the ordinate and the concentration (pg/ml) of the standards on the abscissa (Fig. 2.4.6a.). The concentrations of substance P in unknown samples were read off the standard curve.

2.4.7. Substance P Analyzed by Liquid Chromatography

This is to investigate whether substance P could be one of the components of the middle molecular fractions that were separated from FHF rat plasma by the liquid chromatographies as described in this thesis (Sections 4.3.1. and 4.3.2.). Substance P standard was a product of Boehringer Mannheim Gmbh

(West Germany). A Ø.6 ml sample of normal rat plasma ultrafiltrate was equally divided into 2 aliquots. The first aliquot was added with substance P standard (Ø.3 mg/Ø.3ml), then chromatographed by the G-15 gel permeation column to check the elution volume of substance P. The second aliquot was added with Ø.3 ml saline as the control, and chromatographed in the G-15 column the same way as the sample with added substance P.

Another in vitro experiment was carried out to study the chromatographic behavior of substance P in DEAE anion-exchange chromatography, as well as to test the removal of substance P by activated charcoal. Two batches of solutions were prepared each containing 1.Ø mg substance P standard dissolved in 2.Ø ml Ø.Ø1 M TRIS (pH 8.6, adjusted by Ø.Ø2 N HCl). Petroleum-based bead-type activated charcoal (CAC) (Ø.1 g) was added to one of the solutions. The other solution without charcoal was used as the control. These two solutions were gently mixed at room temperature for 2 hours using a Lab-Line Orbit Environ Shaker (Lab-Line Instruments Inc, Ill, USA). The charcoal beads were carefully removed from the first solution. The two solutions were then chromatographed by the anion-exchange DEAE A-25 columns with a sodium chloride gradient from Ø to 1.Ø M in TRIS buffer. The anion-exchange chromatographic procedures were identical to that described in section 2.4.2. of this thesis.

CHAPTER 3

RESULTS

3.1. RESULTS OF GENERAL PATHOLOGICAL STUDIES

3.1.1. Electron Microscopic Findings of Hepatocellular Injury

Electron microscopic examination indicates severe hepatocellular injury in fulminant hepatic failure rats with grade 3 coma 48 hours after GalN administration (Fig. 3.1.1a. & 3.1.1b.).

In Fig. 3.1.1a. the micrograph depicts a normal hepatocyte with intact ultrastructures of mitochondria, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER) and distinct glycogen rosettes scattered throughout the cytoplasma.

The injury to the hepatocytes is seen in Fig. 3.1.1b. In comparison to the normal hepatocyte, the ultrastructure in FHF cell is dramatically distorted. In the FHF cell the glycogen rosettes have almost completely disappeared. The amount of rough endoplasmic reticulum (RER) is diminished and the remaining RER is dilated. The mitochondria are swollen and much enlarged. The accumulation of lipids in the cytoplasm tends to form large fat droplets which are scattered throughout the hepatocyte. Autophagic vacuoles can be seen which contain remnants of the organelles and the fat droplets.

3.1.2. Body Weight Changes of GalN-FHF Rats

In a group of 60 rats, the body weight was measured before and 48 hours after GalN injection.

The results are shown in Fig. 3.1.2.. All rats used were male Wistar rats weighing 270 - 300 g each. The average body

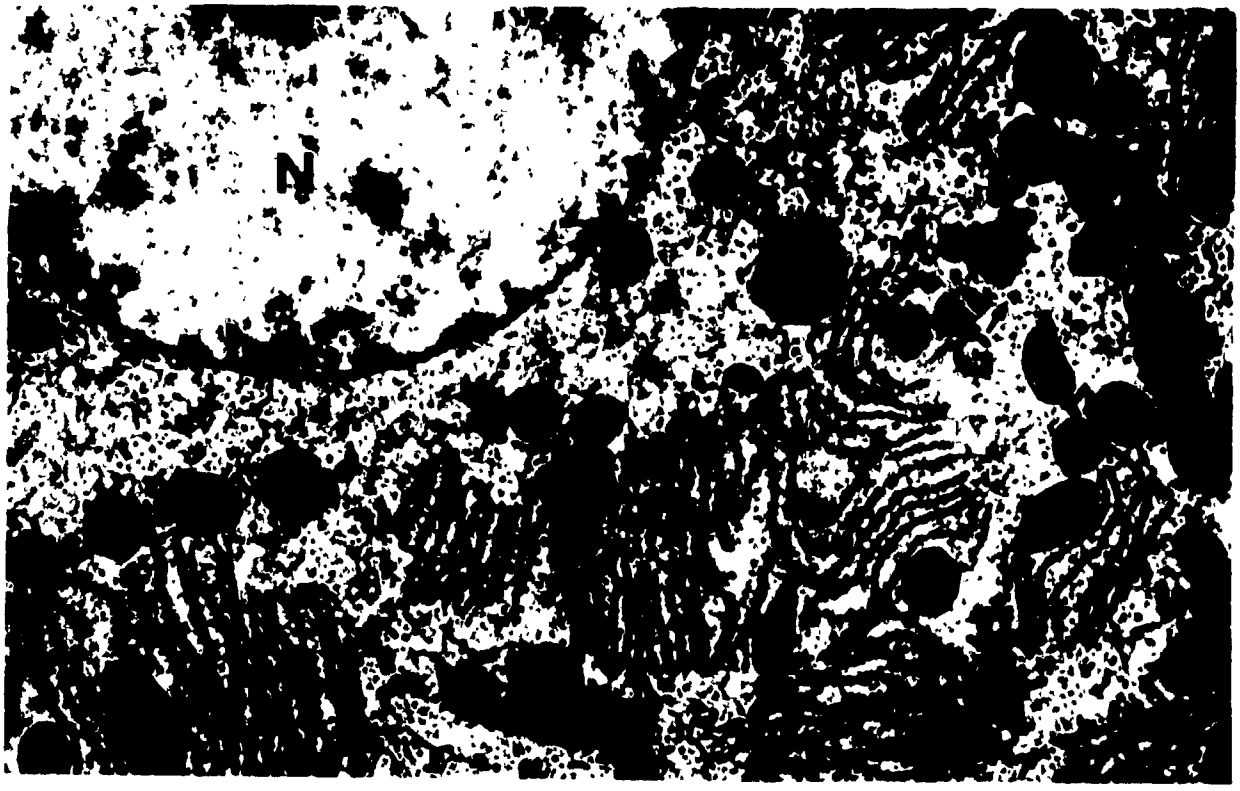


Fig. 3.1.1a. The electron micrograph of a normal hepatocyte. The intact ultrastructures of the nucleus (N), the mitochondria (M), the rough endoplasmic reticulum (R), the smooth endoplasmic reticulum (S), and the glycogen rosettes (G).

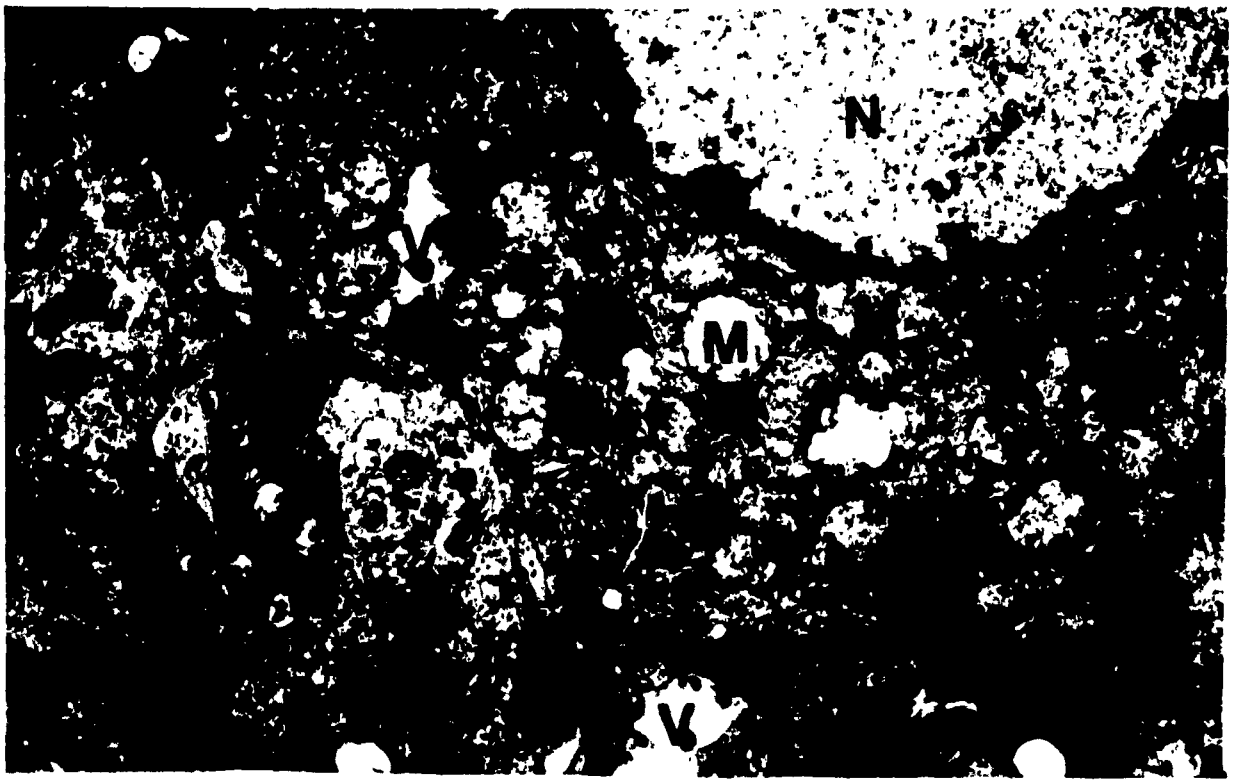


Fig. 3.1.1b. The electron micrograph of a hepatocyte from the liver of a GalN-induced FHF rat (with grade 3 coma). This picture displayed swollen mitochondria (M), accumulation of fat droplets (D) and the autophagic vacuoles (V). The glycogen rosettes are diminished when compared to the normal hepatocyte as shown in Fig. 3.1.1a.

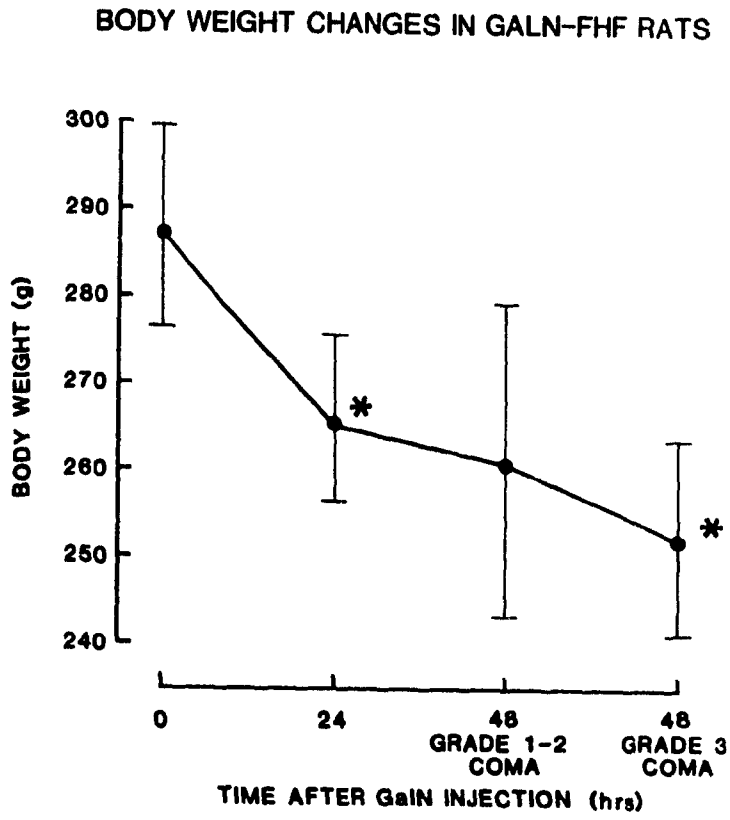


Fig. 3.1.2. Body weight changes of the rats after GalN injection. The body weight of the rats 24 hr and 48 hr (grade 3 coma) was significantly decreased. The * label indicates the statistical significance ($p<0.05$).

weight at the time of GalN injection was 287.2 ± 12.9 g (mean ± S.D.). The average body weight dropped to 265.9 ± 9.4 g 24 hours after GalN administration. Forty-eight hours after GalN injection, 20 rats out of the 60 died. The body weight of the 40 surviving rats was 261.8 ± 18.5 g. Among these 40 rats, 17 were in grade III hepatic coma. Of these 17 rats, the average body weight was 252.6 ± 11.8 g, which was significantly lower than the average of the 40 surviving rats.

When examined with the Student t-test, the loss of average weight of the surviving rats at 24 hrs, and for the 17 rats in grade III hepatic coma 48 hrs after GalN injection were all statistically significant when compared to the average body weight at the time of GalN injection.

The loss of body weight in GalN-FHF rats may be partially due to dehydration. It may also be due to a severe general catabolism in fulminant hepatic failure.

3.1.3. Plasma protein changes in GalN-FHF

The plasma total protein and albumin levels were measured in normal rats, rats at 24 h after GalN administration, and rats in various grades of coma 48 hrs after GalN administration (Fig. 3.1.3.).

In a group of 20 rats, the total plasma protein before the injection of GalN was 5.6 ± 0.3 g/dl (mean ± S.D.). It was significantly decreased to 5.07 ± 0.39 g/dl in rats 24 hrs after GalN injection ($P < 0.05$). At 48 h after GAlN, the total

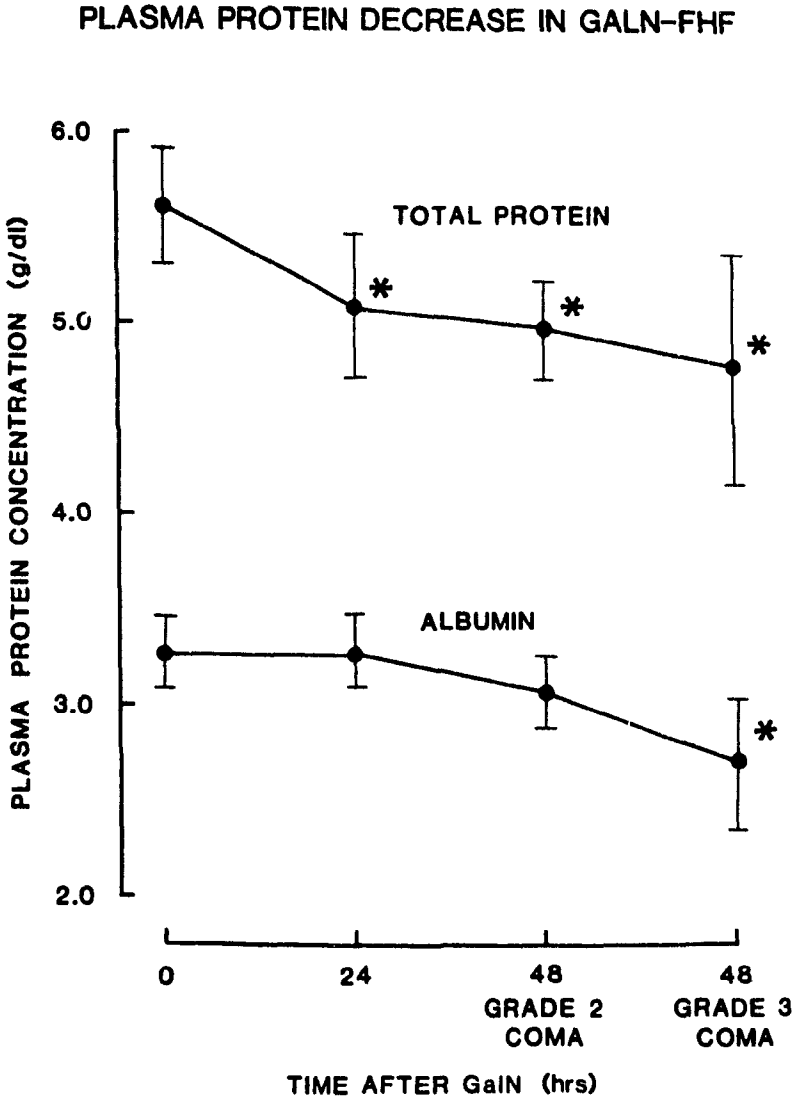


Fig. 3.1.3. The levels of total protein and albumin in plasma in GalN injected rats were significantly decreased in comparison to the levels immediately before GalN injection. The * labels indicate the statistical significance ($p<0.05$).

plasma protein levels were 4.96 ± Ø.259 g/dl in grade II coma rats and 4.75 ± Ø.59 g/dl in grade III coma rats. Both levels were significantly lower than that in normal rats.

The albumin levels in these rats were also decreasing during the course of progressive GalN hepatitis. Albumin level in grade III hepatic coma rats was 2.7 ± Ø.34 g/dl, which was significantly lower than that in rats before GalN injection ($p < Ø.Ø5$). In rats with grade II coma 48 hrs after GalN administration, the albumin level was 3.Ø7 ± Ø.17 g/dl. This level was also lower than the normal but the difference was not significant.

The Decrease of the plasma protein levels are the results of the reduced hepatic synthesis and the increased protein catabolism. This may contribute to the accumulation of nitrogenous intermediate metabolites, such as peptides and amino acids.

3.2. RESULTS OF AMINO ACID CHANGES IN GALN-FHF RATS

3.2.1. Amino Acids in Plasma of Normal and FHF Rats

All the amino acids, except for arginine, were significantly increased in the plasma of FHF rats as compared to the normal rats (Table 3.2.1. & Fig. 3.2.1.). The magnitudes of increase of these amino acids ranged from 2 fold in Glu, Trp and Ile levels, to 22 fold in Orn and 23 fold in Tyr levels. All the differences were statistically significant when examined with Student t-test. Aromatic amino acids (AAA)

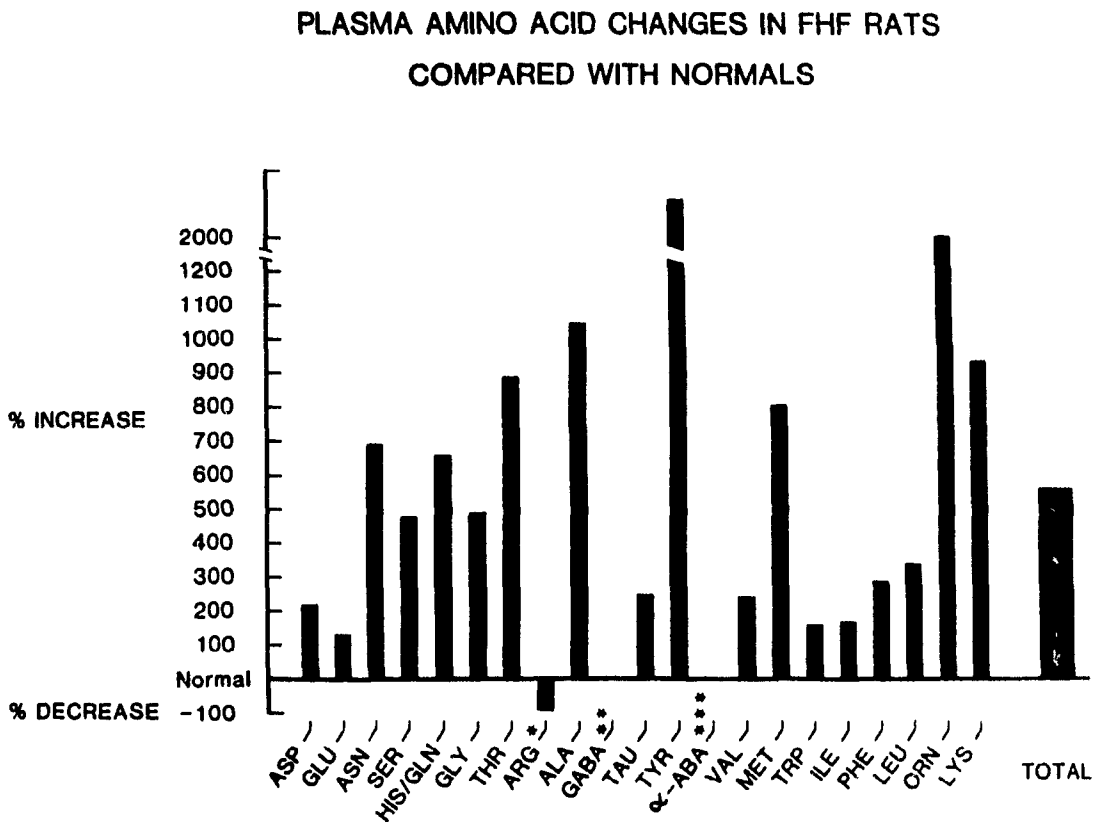


Fig. 3.2.1. The changes of plasma amino acids in FHF rats expressed as the percentage increase or decrease in comparison to the normal levels which are represented by the baseline. The concentration values and the statistical significance are listed in Table 3.2.1.. The total amino acid concentration in FHF plasma was 6.7 times as high as that in the normal plasma.

Table 3.2.1. Plasma amino acids in normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/ml)	FHF rat (n=6) (nmole/ml)	Ratio FHF/normal	P
ASP	14.36 ± 0.10	45.88 ± 22.11	3.19	< 0.05
GLU	95.54 ± 29.19	219.05 ± 94.43	2.29	< 0.05
ASN	50.18 ± 6.84	396.22 ± 57.01	7.90	< 0.001
SER	204.73 ± 15.72	1192.22 ± 184.19	5.82	< 0.001
HIS/GLN	736.64 ±210.81	5602.99 ±1203.02	7.61	< 0.001
GLY	246.62 ± 45.53	1450.92 ± 363.47	5.88	< 0.001
THR	162.92 ± 21.75	1613.29 ± 290.52	9.90	< 0.001
ARG*	116.69 ± 19.67	1.95 ± 2.51	0.02	< 0.001
ALA	512.17 ±104.42	5896.03 ± 557.07	11.51	< 0.005
GABA**				
TAU	181.98 ± 41.54	639.54 ± 223.72	3.51	< 0.005
TYR	61.47 ± 1.86	1433.18 ± 427.65	23.32	< 0.001
α-ABA***		177.20 ± 20.53		
VAL	177.02 ± 15.27	598.58 ± 94.78	3.38	< 0.001
MET	51.11 ± 4.13	463.73 ± 76.72	9.07	< 0.001
TRP	74.92 ± 9.03	190.68 ± 29.90	2.55	< 0.001
ILE	85.49 ± 14.06	228.33 ± 35.79	2.67	< 0.001
PHE	74.26 ± 6.78	288.30 ± 65.46	3.88	< 0.001
LEU	154.22 ± 24.35	519.08 ± 92.91	3.37	< 0.001
ORN	30.18 ± 10.51	659.54 ± 68.50	21.85	< 0.001
LYS	279.00 ± 22.58	2904.22 ± 583.86	10.41	< 0.001
TOTAL	3536.15 ±278.07	23521.83 ±2971.15	6.65	< 0.001

* Arginine was the only amino acid that decreased in the FHF rat plasma.

** GABA was not detected in both normal and FHF plasma.

*** α-Amino-butyric acid was detected in FHF, but not in normal plasma.

increased to a much greater extent than branched chain amino acids (BCAA). Of the AAAs, tyrosine increased by 23 fold, phenylalanine by 4 fold, and tryptophan by 2.5 fold. BCAAs Val, Ile and Leu were increased by 3 to 4 fold. The resultant molar ratio of BCAA to AAA (Val+Leu+Ile / Tyr+Phe) was lowered from 3.Ø7 in the normal to Ø.87 in FHF rats. The arginine level in FHF rats was decreased so markedly that in some subjects it fell below the sensitivity range of the fluorescence detector. The level of α-amino butyric acid (α-ABA) was undetectable in the normal, but in FHF rats it was 177.2 nmol/ml. Methionine level rose from 51.11 $\pm$ 4.13 nmol/ml in the normal to 463.73 $\pm$ 76.72 nmol/ml in FHF animals which represented an increment of 9-fold. GABA was not detected in both normal and FHF rat plasma using the present technique even though the detection sensitivity of the fluorometer was set as high as to detect mere picomoles.

Since His and Gln were co-eluted by HPLC, their concentrations were quantitated as the combination of the two. His/Gln levels were 736.64 $\pm$ 21Ø.81 nmol/ml in normal rats, and 56Ø2.99 $\pm$ 12Ø3.Ø2 nmol/ml in FHF. The elevation was 7.6 times.

3.2.2. Amino Acids in Portal Plasma of Normal And FHF Rats

For both normal and FHF rats, amino acids in portal plasma of rats followed a very similar pattern as amino acids in systemic blood plasma (Table 3.2.2. & Fig. 3.2.2.). All amino acids in portal blood plasma, except Arg, were found increased

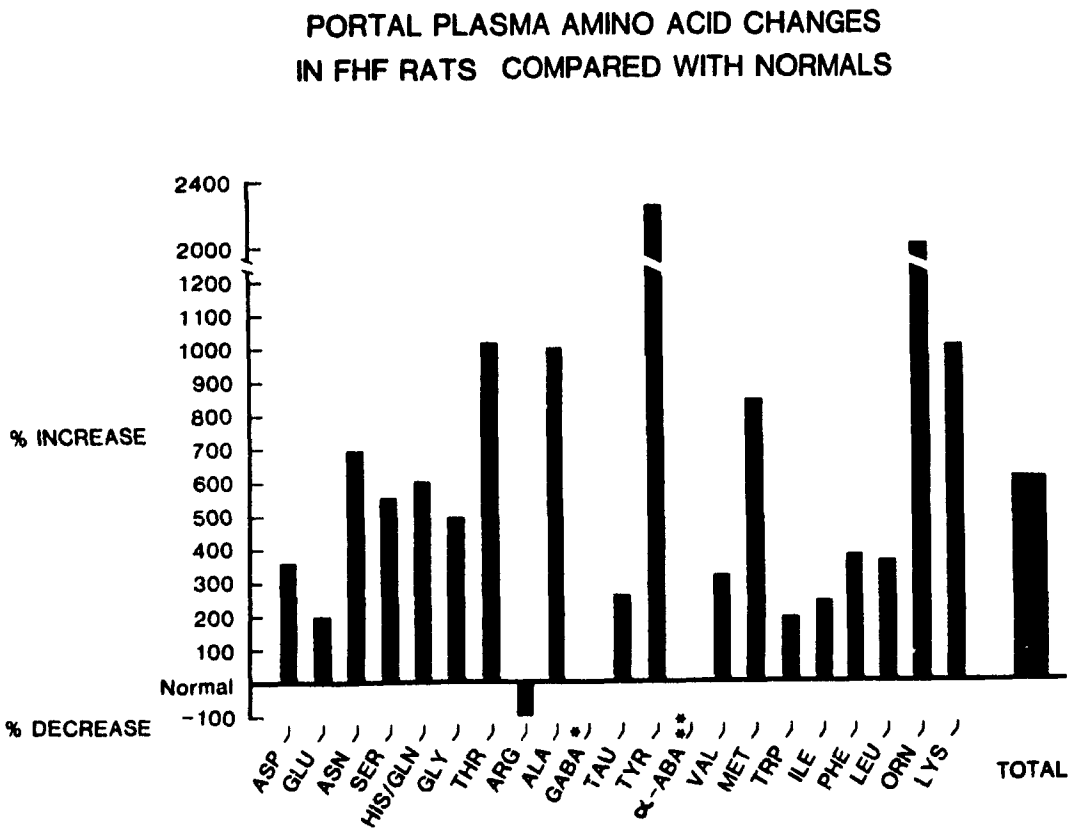


Fig. 3.2.2. The percentage increase or decrease in portal plasma amino acid concentrations are shown in this graph. The average normal levels are represented by the baseline. The concentration values and statistical significance are listed in Table 3.2.2.. The total amino acid concentration in FHF portal plasma was increased by 7 fold.

Table 3.2.2. Portal plasma amino acids in normal and FHF rats

Amino acid	Normal rat (n=3) (nmole/ml)	FHF rat (n=3) (nmole/ml)	Ratio FHF/normal	P
ASP	13.36 ± 2.89	61.44 ± 36.47	4.60	
GLU	120.24 ± 7.90	355.40 ±203.85	2.96	
ASN	45.33 ± 15.04	362.39 ± 73.05	7.99	< 0.001
SER	168.14 ± 43.89	1098.71 ±158.45	6.53	< 0.001
HIS/GLN	631.48 ± 62.01	4448.29 ±687.45	7.04	< 0.001
GLY	248.41 ± 52.93	1455.57 ±546.08	5.86	< 0.001
THR	128.56 ± 22.14	1437.11 ±218.34	11.18	< 0.001
ARG*	89.16 ± 16.48			
ALA	509.98 ±108.87	5614.05 ±558.83	11.01	< 0.001
GABA**				
TAU	194.75 ± 55.93	695.78 ±302.54	3.57	< 0.05
TYR	52.29 ± 9.42	1227.01 ±595.71	23.47	< 0.002
α-ABA***		170.85 ± 17.40		
VAL	149.77 ± 32.16	634.95 ±154.24	4.24	< 0.002
MET	44.36 ± 8.59	417.60 ± 52.48	9.41	< 0.001
TRP	64.44 ± 24.53	190.61 ± 42.46	2.96	< 0.005
ILE	66.44 ± 17.73	221.74 ± 26.13	3.34	< 0.001
PHE	58.46 ± 13.55	278.70 ±110.97	4.77	< 0.02
LEU	120.52 ± 33.00	561.09 ±154.05	4.66	< 0.005
ORN	30.85 ± 10.18	642.30 ±109.42	20.82	< 0.001
LYS	262.59 ± 53.44	2907.30 ±520.79	11.07	< 0.001
TOTAL	3116.28 ±554.34	22165.13 ±3718.78	7.11	< 0.001

* Arginine was not detected in the FHF portal plasma using the present fluorometric technique.

** GABA was not detected in both normal and FHF portal plasma.

*** α-Amino-butyric acid was detected in FHF, but not in normal portal plasma.

in FHF subjects compared to the normals. The incremental magnitudes of AAAs Tyr, Phe, and Trp were 23, 4.8, and 3 fold respectively in FHF portal blood plasma compared to normal levels . These increments were quite close to the increments of Tyr, Phe and Trp (23, 3.9 and 2.6 fold respectively) in systemic blood plasma. The BCAAs Val, Leu and Ile, in FHF portal plasma increased 3-4 fold and did not differ from the incremental magnitudes in systemic plasma. The molar ratio of BCAA/AAA in plasma of portal blood was 3.Ø4 in normal and Ø.94 in FHF rats.

The decrease of Arginine concentration in FHF in portal blood plasma was as significant as in systemic blood plasma. It was virtually undetectable in portal plasma as compared to a mere 1.95 $\pm$ 2.51 nmole/ml in systemic blood plasma.

Thus the total analyzable amino acids in portal plasma were almost identical to those in the systemic blood plasma. This phenomenon is noted in both normal and FHF subjects.

3.2.3. Amino Acids in CSF of Normal and FHF Rats

Most of the amino acids in CSF of FHF rats were found to be substantially increased (Table 3.2.3. & Fig. 3.2.3.). The 17-fold increase of tyrosine was the most marked. Tryptophan and phenylalanine increased by 7 and 4 fold respectively and methionine increased by 6 fold. The concentrations of the BCAAs Val, Ile and Leu were elevated by 2.5 to 3.5 fold, which closely reflected the changes of these amino acids in the plasma. The BCAA/AAA molar ratio dropped from 1.Ø2 in the

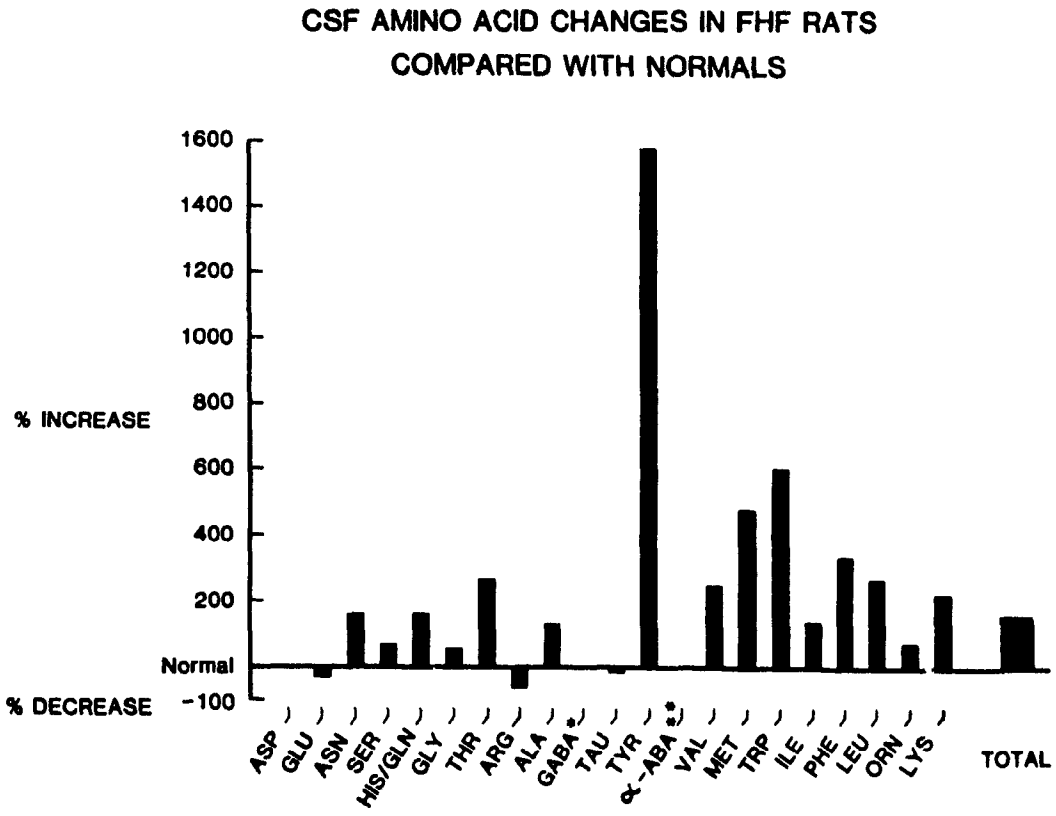


Fig. 3.2.3. Percentage changes in amino acids in FHF cerebral spinal fluids as compared to the normal average levels which are expressed as the baseline. The levels of the individual amino acids and the statistical significance are listed in Table 3.2.3.. The total amino acid concentration in FHF CSF was 2.6 times as high as that in the normal CSF.

Table 3.2.3. CSF amino acids in normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/ml)	FHF rat (n=6) (nmole/ml)	Ratio FHF/normal	P
ASP*				
GLU	10.61 ± 9.78	3.04 ± 3.50	0.28	N.S.
ASN	3.29 ± 0.41	8.58 ± 4.40	2.61	N.S.
SER	69.73 ± 4.53	116.07 ± 18.64	1.66	< 0.005
HIS/GLN	724.00 ±88.96	1915.10 ±222.78	2.65	< 0.001
GLY	7.14 ± 1.00	11.22 ± 9.12	1.57	N.S.
THR	51.47 ± 3.68	190.60 ± 38.69	3.70	< 0.001
ARG**	28.11 ± 1.28	11.37 ± 3.08	0.40	< 0.001
ALA	58.91 ± 7.51	138.76 ± 42.23	2.36	< 0.02
GABA*				
TAU	27.90 ± 5.61	26.41 ± 21.54	0.95	N.S.
TYR	6.10 ± 1.01	102.25 ± 36.83	16.76	< 0.005
α-ABA***		8.56 ± 0.58		
VAL	3.78 ± 0.47	13.27 ± 2.92	3.51	< 0.002
MET	5.09 ± 0.29	29.33 ± 8.05	5.76	< 0.002
TRP	2.00 ± 0.90	14.08 ± 3.24	7.04	< 0.001
ILE	2.07 ± 0.50	5.04 ± 1.09	2.43	< 0.005
PHE	4.00 ± 1.17	17.53 ± 5.34	4.38	< 0.005
LEU	4.46 ± 1.16	16.37 ± 3.91	3.67	< 0.002
ORN	4.51 ± 2.75	7.76 ± 3.16	1.72	N.S.
LYS	67.67 ± 5.21	218.84 ± 40.65	3.23	< 0.001
TOTAL	1014.96 ±94.54	2656.45 ±385.03	2.62	< 0.001

* ASP and GABA were not detected in both normal and FHF CSF.

** Arginine was significantly decreased in the FHF CSF.

*** α-Amino-butyric acid was detected in FHF, but not in normal CSF.

normal to an abnormal low value of 0.29 in FHF.

In FHF rats, the increases of amino acid concentrations in CSF were generally less marked in comparison to blood plasma. Despite the drastic increase in plasma amino acid levels, the individual amino acids in CSF, except arginine and His/Gln, only accounted for a mere 1-12% of the respective plasma levels in FHF rats. In normal rats, the CSF amino acid levels were only 2-34% of the plasma levels. For most individual amino acids in FHF animals, their increases in CSF were far less than that found in the plasma. This was reflected by the ratio of CSF/plasma amino acids. The values of this CSF/plasma ratio in FHF were smaller than those in normal animals for most amino acids.

Again, the arginine level was significantly decreased in CSF. But unlike the virtual disappearance of arginine from the plasma, arginine in CSF was reduced by 60%. In normal rats, the combined concentration of His/Gln in CSF was almost the same as that in the plasma. In FHF rats, however, regardless of 7.6-fold increase in plasma, CSF His/Gln concentration increased only by 2.7 fold. Thus, the CSF/plasma ratio of His/Gln was 1 in normal rats, and only 0.34 in FHF rats.

Several amino acids did not show any significant changes in CSF. These included Asn, Gly, Tau and Orn. Glutamic acid decreased slightly, but not significantly, in FHF cerebrospinal fluid. Other amino acids, including Ser, Thr, Ala, Met and Lys, all increased by 2 to 6 fold in FHF CSF when compared to normal levels.

3.2.4. Amino Acids in the Brains of Normal and FHF Rats

The increase in most amino acids in the brain hemisphere (including cerebrum, diencephalon and mesencephalon) of FHF rats was quite significant although not as drastic as what was seen in the FHF plasma (Table 3.2.4. & Fig. 3.2.4.).

Tyrosine was found to have increased the most as its level increased by 6 fold from 147.73 ± 20.84 in the normal to 885.17 ± 258.58 nmol/g in the FHF rats. Trp and Phe concentrations in the FHF rat brain rose by 2.5 and 1.5 fold respectively. The increase of methionine in FHF brain was 2.6 fold. The increases of these 4 amino acids, and a few others, were the most significant. The BCAAs Val, Ile and Leu all increased to about 1.5 times the normal level. Lysine and threonine rose by 2.8 and 2.5 fold respectively. Those amino acids that increased in FHF brains by 2 to 3 fold included His/Gln, Gly and Ala. The levels of Asp, Glu, Asn, Ser, GABA, Tau and Orn did not show any significant change.

Although GABA level in the brain hemisphere sample showed only very slight, but not significant increase, the concentrations in certain areas of the brain did show some significant changes. This will be dicsussed in the Section 3.2.8. of this chapter. In normal rat brain, α-ABA was not detected, but in the brains of FHF animals, α-ABA rose considerably to 124.44 ± 28.37 nmol/g, which was comparable to some of the neutral amino acids in the brain, e.g., Trp and Ile.

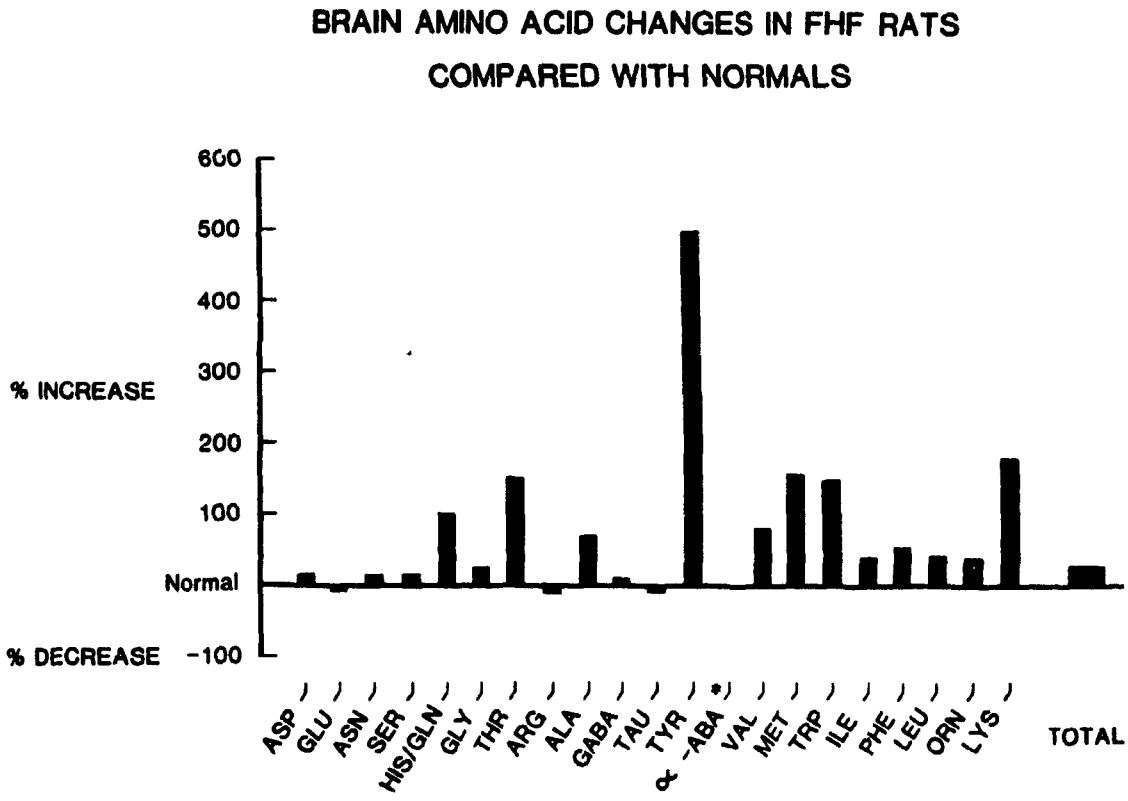


Fig. 3.2.4. Percentage changes in amino acid concentrations in FHF brain hemisphere as compared to the normal baseline levels. For descriptive notes see Table 3.2.4.. The total concentration of the amino acids in the FHF brain increased by 27% in comparison to the normal levels.

Table 3.2.4. Amino acids in brain hemispheres of normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/g)	FHF rat (n=6) (nmole/g)	Ratio FHF/normal	P
ASP	5725.17 ± 437.40	6664.20 ±3184.99	1.16	
GLU	8091.86 ± 339.60	7854.72 ± 646.62	0.97	
ASN	143.66 ± 4.52	163.83 ± 21.28	1.14	
SER	1541.25 ± 146.72	1745.40 ± 136.94	1.13	
HIS/GLN	7390.05 ± 29.47	14924.65 ±4851.13	2.02	< 0.02
GLY	2426.92 ± 410.70	2998.94 ± 238.77	1.24	< 0.05
THR	638.36 ± 110.28	1616.92 ± 106.17	2.53	< 0.001
ARG	300.44 ± 63.50	280.46 ± 73.07	0.93	
ALA	1438.02 ± 220.13	2431.37 ± 616.34	1.69	< 0.02
GABA	4246.03 ± 655.94	4616.87 ± 489.44	1.09	
TAU	6828.17 ± 220.34	6630.77 ± 425.94	0.97	
TYR	147.73 ± 20.84	885.17 ± 258.58	5.99	< 0.001
α-ABA*		124.44 ± 28.37		
VAL	190.63 ± 20.63	339.33 ± 49.54	1.78	< 0.001
MET	119.85 ± 18.96	309.50 ± 32.87	2.58	< 0.001
TRP	55.64 ± 18.13	137.08 ± 32.67	2.46	< 0.005
ILE	127.73 ± 11.10	176.99 ± 25.18	1.39	< 0.01
PHE	172.59 ± 25.90	263.70 ± 28.90	1.53	< 0.001
LEU	294.11 ± 47.67	424.44 ± 63.18	1.44	< 0.01
ORN	66.43 ± 25.33	93.98 ± 44.20	1.41	
LYS	352.22 ± 80.82	985.24 ± 194.29	2.80	< 0.001
TOTAL	43518.89 ±2300.50	55210.78 ±2879.25	1.27	< 0.001

* α-Amino-butyric acid (α-ABA) was detected in FHF brain hemisphere, but not in the normals.

The molar ratio of BCAA/AAA was reduced from 1.91 in normal brain to 0.82 in FHF brain. The reduction of BCAA/AAA ratio was mostly due to the 6-fold increase of tyrosine.

3.2.5. Amino Acids in the Liver of Normal and FHF Rats

The liver in fulminant hepatic failure would be expected to display some significant changes in its amino acid contents. Indeed, quite a few amino acids were found to be significantly increased (Table 3.2.5. & Fig. 3.2.5.). Among them, GABA concentration in FHF was twice that in the normal. The increase in Tyr concentration, which was slightly less than 2 fold, was the second greatest among the increased amino acids in FHF liver. Trp concentration increased by 60% in FHF. Phe did not show any difference in the concentrations between the normal and FHF livers. None of the three branched chain amino acids displayed any significant change in their concentrations in the FHF liver. The molar ratio of BCAA/AAA was reduced from 2.46 in the normal to 1.59 in the FHF liver.

Unexpectedly, the relatively less important amino acid α-ABA had a concentration in FHF liver 11 times that in the normal liver. Glutamic acid, threonine, and alanine in the liver were found significantly increased in FHF animals. The magnitudes of these increases ranged only from 27% to 50%, which were just great enough to yield statistical significance.

No other amino acids were found to have any significant differences in their concentrations between the normal and FHF livers. These included Asp, Asn, Ser, His/Gln, Gly, Tau, Met,

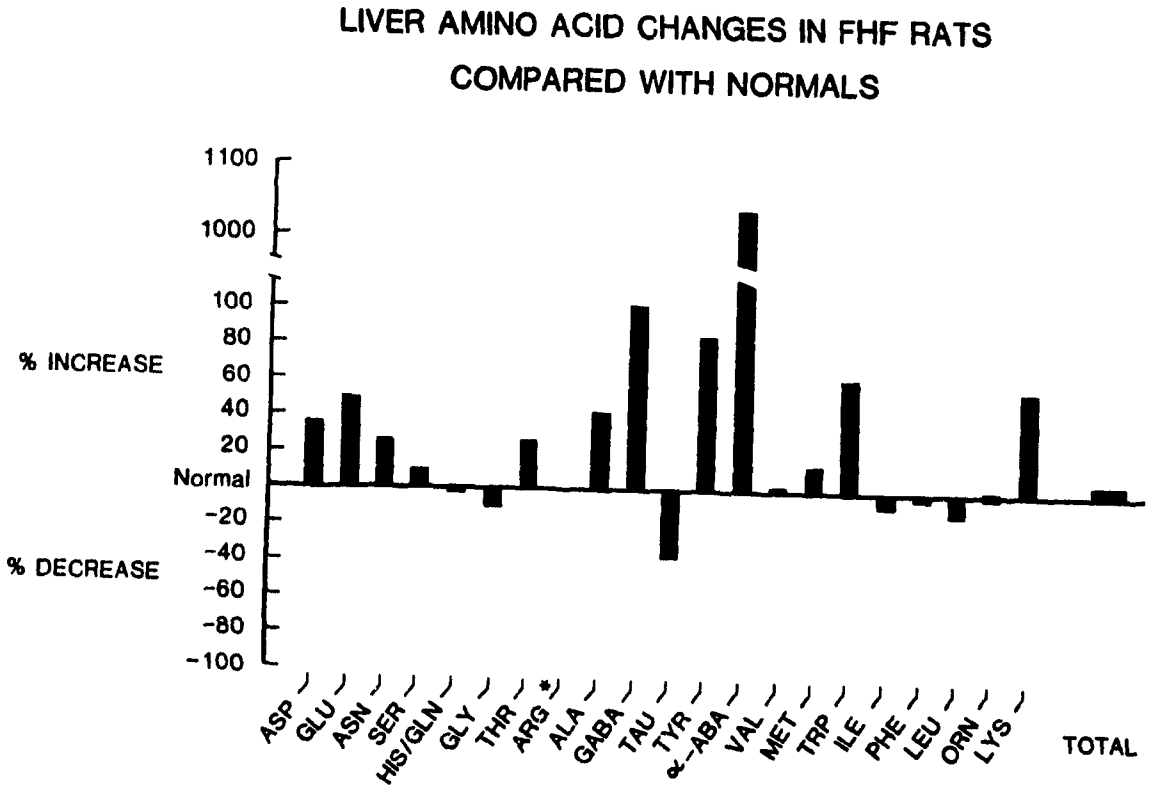


Fig. 3.2.5. The amino acid changes in the FHF liver expressed as percentage increase or decrease in comparison to the normal baseline levels. The total increase of amino acids in FHF liver is 6%. Other descriptions are given in Table 3.2.5..

Table 3.2.5. Amino acids in livers of normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/g)	FHF rat (n=6) (nmole/g)	Ratio FHF/normal	P
ASP	1407.78 ± 338.81	1918.98 ± 519.46	1.36	
GLU	3117.21 ± 740.73	4674.19 ± 739.94	1.50	< 0.05
ASN	941.60 ± 263.40	1194.49 ± 179.94	1.27	
SER	3150.46 ± 331.29	3458.23 ± 497.33	1.10	
HIS/GLN	9307.04 ±1927.63	9241.18 ±1991.92	0.99	
GLY	5980.05 ± 860.56	5417.27 ± 992.76	0.91	
THR	2203.69 ± 219.74	2803.86 ± 390.28	1.27	< 0.05
ARG*				
ALA	6733.54 ± 1507.95	9620.21 ± 888.92	1.43	< 0.01
GABA	235.20 ± 139.13	504.36 ± 117.81	2.14	< 0.02
TAU	6017.13 ±3292.67	3767.01 ± 575.29	0.63	
TYR	1187.53 ± 224.19	2198.24 ± 453.83	1.85	< 0.005
α-ABA	21.25 ± 4.07	242.46 ± 28.80	11.41	< 0.001
VAL	1940.41 ± 334.04	1957.92 ± 246.61	1.01	
MET	851.37 ± 118.93	976.40 ± 133.18	1.15	
TRP	228.85 ± 36.25	370.78 ± 49.50	1.62	< 0.002
ILE	1011.01 ± 198.25	950.18 ± 136.42	0.94	
PHE	1008.43 ± 119.48	986.32 ± 124.59	0.98	
LEU	2455.08 ± 323.64	2160.84 ± 243.69	0.88	
ORN	1647.28 ± 167.02	1641.05 ± 326.56	1.00	
LYS	2481.13 ± 338.00	3902.81 ±1552.79	1.57	
TOTAL	59443.39 ±9025.41	62867.44 ±8060.05	1.06	

* Arginine was not detected in either normal or FHF livers using the present technique.

Orn, and Lys. The 6% increase in the total concentration of free amino acids in FHF liver was not statistically significant.

It is interesting to note that arginine was undetectable in both normal and FHF livers.

In general, the changes found in the liver of fulminant hepatic failure animal model was relatively mild in comparison to the changes in blood plasma, CSF and brain.

3.2.6. Amino Acids in Skeletal Muscle of Normal and FHF Rats

In fulminant hepatic failure, the skeletal muscle showed an almost universal rise in its amino acid contents (Table 3.2.6. & Fig. 3.2.6.)

The amino acid with the greatest increase in magnitude was α-ABA, which rose by 15 fold. Tyrosine, with its 6.7-fold increment, had the second greatest increase. Trp and Phe levels rose by 4 and 2 fold respectively. The three BCAAs, Val, Leu, and Ile, all increased by 2 to 2.5 fold. As a result of the uneven changes in AAA and BCAA concentrations, the BCAA/AAA molar ratio was reduced from 1.68 to 0.88.

Methionine level rose by 3.7 fold. The amino acids Asp, Asn, Ser, Thr, Ala, Orn and lys all increase significantly by 2 to 4 fold.

The combined concentration of His/Gln was increased by 60% in FHF, but due to a large discrepancy, did not reach the statistical significance in Student t-test. Glycine and taurine were found to have no change in their concentrations in

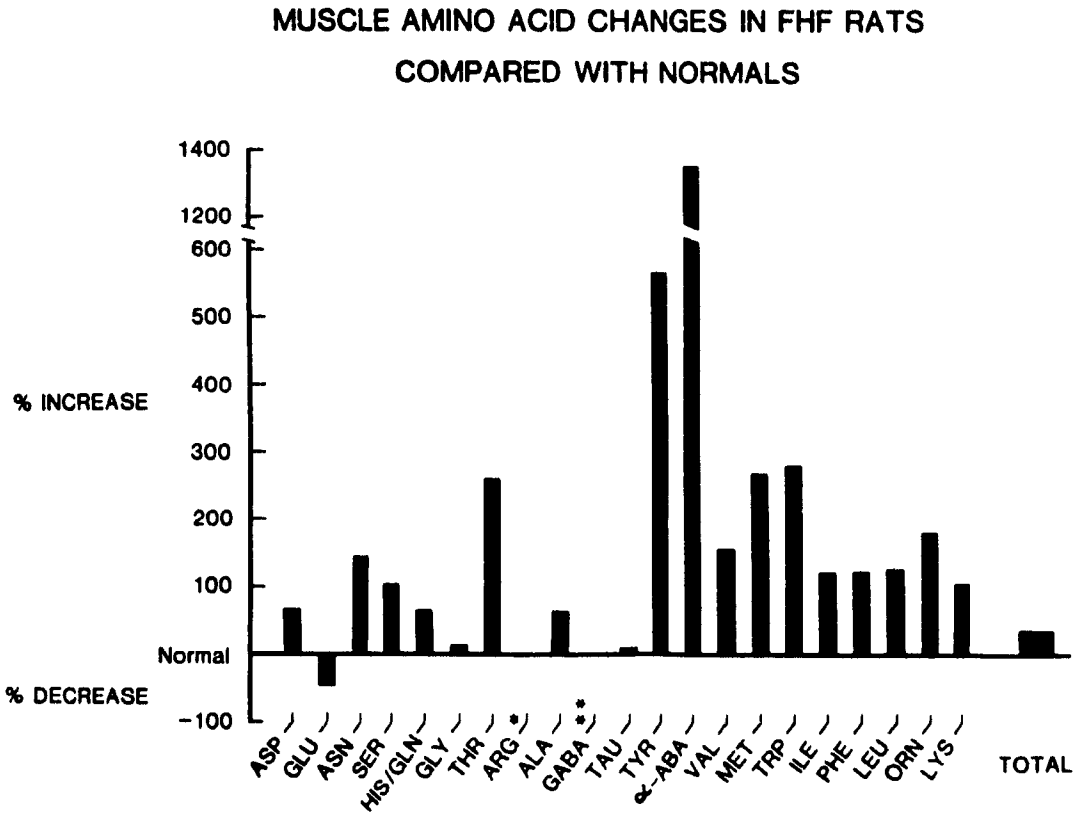


Fig. 3.2.6. The changes in amino acids in the skeletal muscle of FHF rats. The percentage increase or decrease of amino acids are compared with the baseline normal levels. Other descriptions are given in Table 3.2.6..

Table 3.2.6. Amino acids in muscles of normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/g)	FHF rat (n=6) (nmole/g)	Ratio FHF/normal	P
ASP	311.82 ± 108.91	521.26 ± 88.92	1.67	< 0.02
GLU	1089.91 ± 136.11	578.43 ± 74.71	0.53	< 0.001
ASN	223.66 ± 35.94	551.30 ± 156.55	2.46	< 0.005
SER	937.09 ± 99.94	1942.44 ± 506.07	2.07	< 0.005
HIS/GLN	5994.89 ±766.96	9979.76 ±3491.08	1.66	
GLY	2703.01 ±465.11	3223.36 ± 548.23	1.19	
THR	558.08 ± 64.21	2000.65 ± 431.71	3.58	< 0.001
ARG*				
ALA	7829.40 ±480.05	12813.16 ± 933.31	1.64	< 0.02
GABA*				
TAU	14046.43 ±778.00	14310.52 ± 544.55	1.02	
TYR	268.50 ± 21.00	1798.32 ± 546.33	6.70	< 0.001
α-ABA	12.43 ± 1.46	180.70 ± 37.93	14.54	< 0.001
VAL	296.11 ± 12.63	773.82 ± 96.51	2.61	< 0.001
MET	174.74 ± 18.38	647.20 ± 145.03	3.70	< 0.001
TRP	68.20 ± 11.88	261.29 ± 35.00	3.83	< 0.001
ILE	187.22 ± 8.13	421.96 ± 56.39	2.25	< 0.001
PHE	257.82 ± 13.62	580.88 ± 110.42	2.25	< 0.001
LEU	399.08 ± 18.17	900.67 ± 120.91	2.26	< 0.001
ORN	50.81 ± 18.89	144.45 ± 36.34	2.84	< 0.005
LYS	745.11 ±107.09	1534.76 ± 406.35	2.06	< 0.01
TOTAL	46405.06 ±1531.77	63478.46 ±6957.10	1.37	< 0.002

* Arginine and GABA were not detected in either normal or FHF rat muscles using the present technique.

skeletal muscle in FHF.

It should be emphasized that neither in the normal, nor in the FHF rats could GABA be detected in the skeletal muscle. Arginine was another amino acid that could not be detected in skeletal muscle of both normal and FHF animals. It is also interesting to note that glutamic acid was the only amino acid that significantly decreased among amino acids in the FHF muscle. A 50% reduction of Glu was highly significant in statistical analysis with Student t-test.

3.2.7. Amino Acids in the Kidneys of Normal and FHF Rats

There were 12 amino acids, among the whole array of 22, showing significant increases in the kidneys of fulminant hepatic failure rats (Table 3.2.7. & Fig. 3.2.7.).

The level of α-ABA in FHF kidney was drastically increased by 47 fold. The other 11 amino acids were increased by only 20% to 80% in the FHF kidney. These 11 amino acids were Glu, His & Gln, Gly, Thr, Ala, GABA, Tau, Tyr, Trp and Orn. The increases in tyr and Trp were only 50 to 60%, which were statistically significant but much less marked than their changes in the other compartments studied. Phenylalanine showed no change in the FHF kidney. The mere 10% increases of the three BCAAs in the FHF kidney, were found insignificant. The BCAA/AAAmolar ratio was 2.97 in FHF and 2.40 in the normal. The reduction in the molar ratio was small and insignificant. The total increase in the FHF kidney amino acids was 21% in comparison to the normal value.

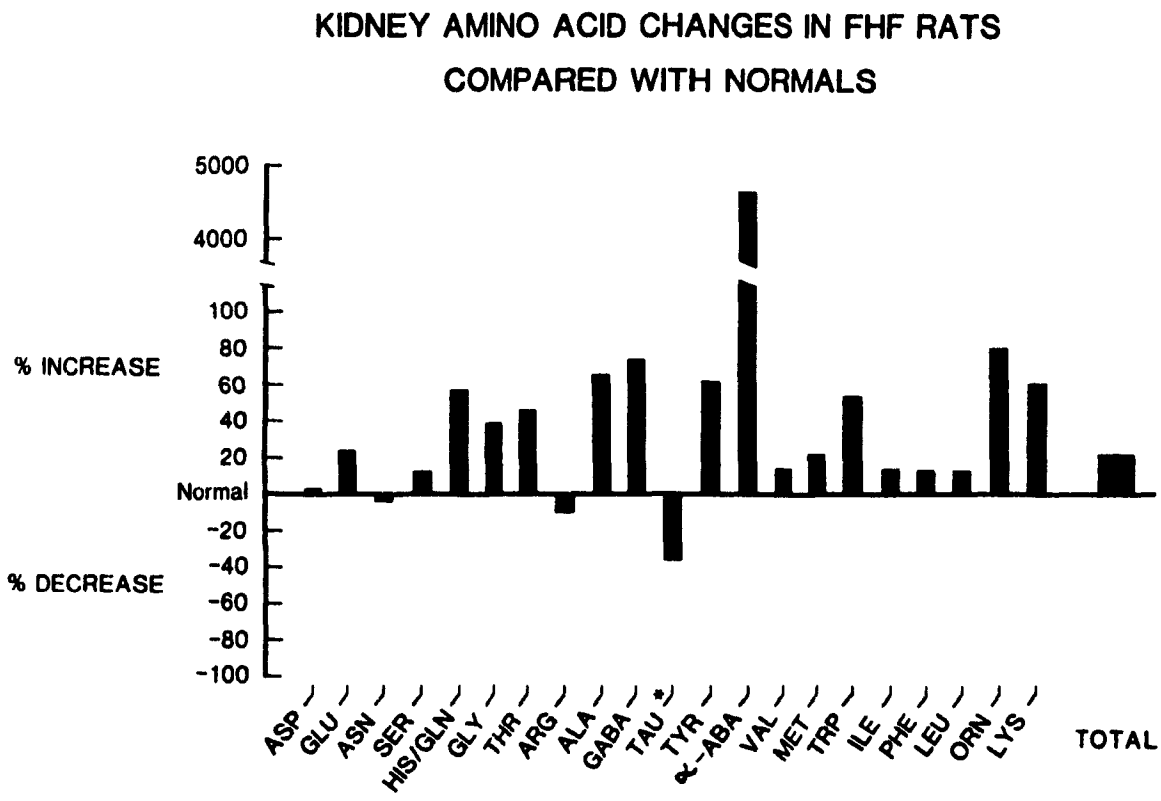


Fig. 3.2.7. The changes of kidney amino acids in FHF rats are expressed as the percentage increase or decrease in relation to the normal baseline levels. The total amino acid concentration was increased by 21% in FHF kidneys. Taurine is the only amino acid that significantly decreased.

Table 3.2.7. Amino acids in kidneys of normal and FHF rats

Amino acid	Normal rat (n=4) (nmole/g)	FHF rat (n=6) (nmole/g)	Ratio FHF/normal	P
ASP	6773.57 ± 397.34	6935.69 ± 1460.83	1.02	
GLU	10903.01 ± 768.35	13513.43 ± 1527.04	1.24	< 0.02
ASN	2722.09 ± 407.60	2629.76 ± 408.58	0.97	
SER	8947.30 ± 703.93	10032.47 ± 1917.01	1.12	
HIS/GLN	3824.47 ± 257.59	6007.95 ± 1568.37	1.57	< 0.05
GLY	11158.16 ±1104.59	15358.65 ± 2119.96	1.38	< 0.01
THR	5790.74 ± 587.21	8473.93 ± 2171.46	1.46	< 0.05
ARG	3690.19 ± 336.62	3323.77 ± 975.93	0.90	
ALA	9434.99 ±1057.81	15564.49 ± 2427.46	1.65	< 0.005
GABA	743.06 ± 128.33	1283.27 ± 597.76	1.73	< 0.05
TAU*	11229.88 ±1658.50	7155.55 ± 1785.87	0.64	< 0.01
TYR	2575.57 ± 151.25	4174.86 ± 832.43	1.62	< 0.01
α-ABA	24.73 ± 10.52	1170.80 ± 339.23	47.34	< 0.001
VAL	5264.12 ± 423.41	6003.80 ± 1216.35	1.14	
MET	2262.94 ± 166.73	2749.97 ± 569.00	1.22	
TRP	581.92 ± 25.35	898.34 ± 168.51	1.54	< 0.01
ILE	2872.48 ± 271.48	3235.94 ± 715.07	1.13	
PHE	2495.86 ± 130.13	2850.89 ± 524.05	1.14	
LEU	6923.48 ± 499.84	7752.39 ± 1516.60	1.12	
ORN	1040.74 ± 69.46	1872.40 ± 467.66	1.80	< 0.01
LYS	5848.66 ± 599.32	9408.43 ± 3324.72	1.61	
TOTAL	114733.19 ±9080.28	138995.98±23560.72	1.21	< 0.002

* Taurine was significantly decreased in FHF rat kidneys.

Table 3.2.8.

GABA IN NORMAL AND FHF RATS

(nmole/g wet tissue)*

Tissue	Normal	FHF	Statistical Significance
Plasma**	nondetectable	nondetectable	N.S.
CSF**	nondetectable	nondetectable	N.S.
Muscle**	nondetectable	nondetectable	N.S.
Liver	235.2 ± 139.1	504.4 ± 117.8	P < 0.02
Kidney	743.1 ± 128.3	1283.3 ± 597.8	P < 0.05
Brain cortex	5281.9 ± 154.3	6821.5 ± 907.8	P < 0.05
Brain stem	3569.4 ± 53.6	4045.6 ± 273.2	P < 0.05
Cerebellum	3522.1 ±1282.4	3381.9 ± 621.3	N.S.
Mid Brain	6759.1 ± 387.3	6757.7 ± 203.0	N.S.

* All values were expressed as mean ± 1 S.D. of a group of 3 samples.

** GABA in plasma, CSF and skeletal muscle was not detected in bothnormalandFHFratsusingthepresentHPLC techniques.

No significant change was found in the FHF kidney contents of Asp, Asn, Ser, Arg, Val, Met, Ile, Phe, Leu and Lys.

3.2.8. Changes of gamma-amino butyric acid (GABA)

In the FHF cerebral hemisphere, GABA concentration is found to be slightly but insignificantly elevated (Table 3.2.4.).

Nevertheless, when carefully dissected brain regions were examined, GABA concentrations were found significantly increased in the cerebrum and the brain stem, but not in the diencephalon and cerebellum. This is a very interesting finding as GABA is the principal inhibitory neurotransmitter in the mammalian brain.

GABA was not detectable in either normal or FHF plasma in GalN FHF rats using the present technique. There was also no detectable amount of GABA in the skeletal muscle. However, GABA concentrations were significantly increased by about 2 fold in both the liver and the kidneys ($p<0.02$ and $p<0.05$, respectively).

3.2.9. Changes of free and total tryptophan in FHF Rats

The significant elevation of total tryptophan (free and protein-bound) concentrations was seen in all body fluids and organ tissues in this study. Total tryptophan was measured and compared between FHF and normal rats in plasma, CSF, brain, liver, muscle and kidney. The level increased by 2.5 fold in FHF plasma, 7 fold in the CSF, about 2.5 fold in the brain

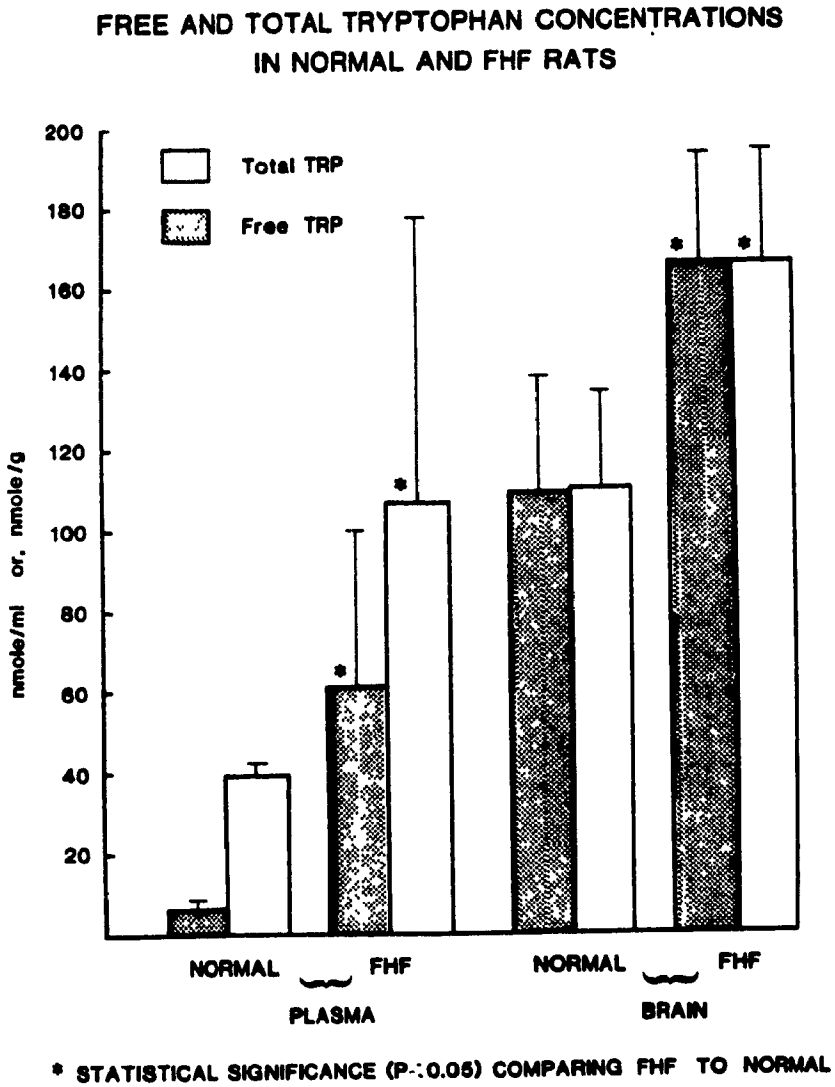


Fig. 3.2.9. Free and total tryptophan concentrations in plasma and brain hemisphere samples in normal and FHF rats. Total tryptophan includes free and protein-bound tryptophan. The * label indicates the significant difference between the normal and FHF subjects.

Table 3.2.9a. Free and Total Tryptophan Concentration Changes in plasma

(nmole/ml, Mean ± 1 S.D.)

	Free TRP	Total TRP	Calculated Bound TRP	% of Free/Total
Normal	5.93 ± 1.07	39.35 ± 3.12	33.42	15.1%
FHF	60.49 ±42.55	106.66 ±72.13	46.17	56.7%
t-Test	P<0.05	P<0.05		P<0.05
Ratio FHF/Normal	10.27	2.71	1.38	3.75

Table 3.2.9b. Free and Total Tryptophan Concentration Changes in Brain Hemisphere

(nmole/g, Mean ± 1 S.D.)

	Free TRP	Total TRP	Calculated Bound TRP	% of Free/Total
Normal	109.6 ± 29.3	109.8 ± 26.0	0.19	99.8%
FHF	166.7 ± 27.8	167.9 ± 29.0	0	99.3%
t-Test	P<0.05	P<0.05		
Ratio FHF/Normal	1.52	1.53	0	

hemisphere, 4 fold in the skeletal muscle, 1.6 fold in the liver, and 1.5 fold in the kidney (Tables 3.2.1. - 3.2.7.). All these increases in total tryptophan concentrations were statistically significant when examined with the Student t-test.

Since it is the concentration of free tryptophan in plasma rather than that of total tryptophan that has been suggested to influence the brain tryptophan levels (Tricklebank et al, 1978), the further investigation of the changes of both total and free tryptophan in GalN-FHF was carried out.

In a group of 5 normal rats and a group of 5 FHF rats, free and total tryptophan concentrations were analyzed with HPLC. The results showed that in normal rats, free (or unbound) tryptophan accounts for only 15% of the total tryptophan concentration in plasma (Table 3.2.9a. and Fig. 3.2.9.). In FHF rat plasma, there was a 10-fold increase in free tryptophan, and the calculated albumin-bound tryptophan in FHF rat was only 1.38 times that of the normal. Thus the 2.7-fold increase in total tryptophan was firstly due to the increase in free tryptophan and secondly due to the protein-bound tryptophan. During this time, the level of free tryptophan was increased to 56.7% of the total. The increases of free, and total tryptophan, and the percentage of free in total tryptophans were all proved statistically significant.

In the brain, it was shown that tryptophan existed almost exclusively in the free form (Table 3.2.9b. and Fig. 3.2.9.). The calculated protein-bound tryptopan was nearly zero in the

brain. The increase of the total tryptophan in FHF brain was found to be entirely due to the increase of the free tryptophan. The final concentration of the free tryptophan was almost identical to that of the total tryptophan, in either normal or FHF rat brains.

In the brain regions other than the cerebrum, the total tryptophan was measured and compared between the normal and FHF subjects. It was shown that the total tryptophan in diencephalon was significantly increased in FHF rats. There was some increase in the total tryptophan concentration in the cerebellum, but the increase was not significant ($0.05 < P < 0.1$) due to a large standard deviation. No significant change in tryptophan concentration in brain stem was shown.

3.2.10. The Correlative Increase in Tyrosine in the Serum, Liver and Brain of FHF Rats

A total of 31 rats were used for the analysis of serum, liver and brain tyrosine changes. This group included 5 normal rats and 4 rats in grade 1, 10 rats in grade 2, 8 rats in grade 3, and 4 in grade 4 hepatic coma.

Figure 3.2.10a. shows that the increases in plasma, liver and brain tyrosine concentrations were associated with the progression of hepatic coma from normal rats to rats in various grades of hepatic coma. The tyrosine concentration was 0.16 ± 0.03 micromole/ml in the normal rat serum, increased to 0.35 ± 0.25 micromole/ml in grade 1 coma; 0.88 ± 0.44 micromole in grade 2 coma; 1.24 ± 0.56 micromole/ml in grade 3 and $1.59 \pm$

Ø.39 micromole/ml in grade 4 hepatic coma. It also showed that tyrosine concentrations in the brain and liver were raised in correlation to the serum tyrosine increase. Tyrosine concentration was Ø.2Ø ± Ø.Ø3 micromole/g brain tissue in the normal rats, increased to Ø.47 ± Ø.13 micromole/g in grade 1 coma; Ø.55 ± Ø.2Ø micromole/g in grade 2; and Ø.8Ø +Ø.33 micromole/g in grade 3 and Ø.8Ø ± Ø.31 micromole/g in grade 4 hepatic coma rats. The concentrations of tyrosine in the plasma and brain were proved to be linearly correlated (r=Ø.6958, p<Ø.Ø5) (Fig. 3.2.1Øb.). Tyrosine concentration in the liver tissue also displayed a pattern of progressive increase which corresponded well to the severity of hepatic failure from normal rats to rats in various grades of hepatic coma.

In the normal liver, tyrosine concentration was Ø.55 ± Ø.13 μmol/g. The level progressively increased in various grades of coma. It was 1.29 ± Ø.31 μmol/g in grade 1, 1.32 ± Ø.62 μmol/g in grade 2, 1.75 ± Ø.55 μmol/g in grade 3 and 1.7Ø ± Ø.22 μmol/g in grade 4 hepatic coma. The concentration of tyrosine in the liver correlated significantly with the tyrosine in the serum (r=Ø.8667, p<Ø.Ø1).

Figure 3.2.1Øb. also depicts the regression curve of the correlative increases of tyrosine in the blood plasma and brain compartments. This curve clearly indicates two facts: (1) brain tyrosine level is elevated when the plasma level rises; (2) the increase in brain tyrosine level is considerably smaller than the increase in the plasma tyrosine. It is

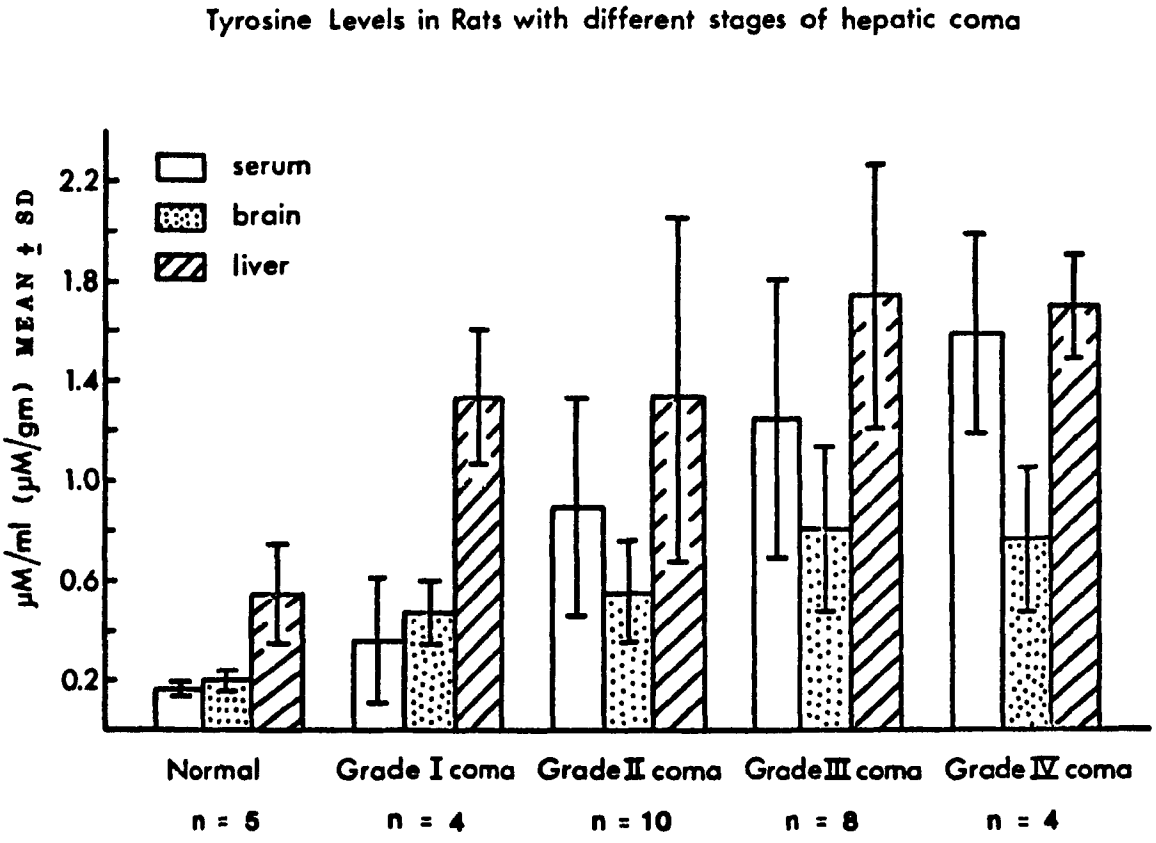


Fig. 3.2.10a. Tyrosine levels in serum, liver, and brain of normal and FHF rats. All serum and brain tyrosine levels in various grades of hepatic coma rats were significantly higher than the normal rats.

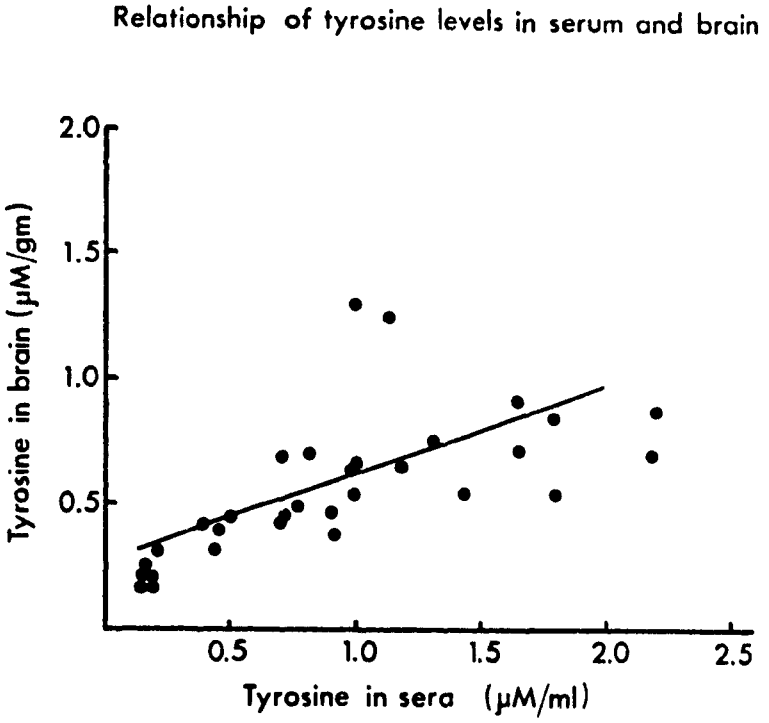


Fig. 3.2.10b. The correlation of tyrosine levels in the serum and brain ($r=0.6968$, $p<0.005$).

noteworthy that brain and liver tyrosine did not increase further beyond grade 3 coma, although plasma tyrosine increased by 25% in grade 4 coma when compared to grade 3 coma.

3.2.11. Amino Acid Distribution in Various Tissue Compartments in Normal and FHF Rats

As shown in the tables and figures in sections 3.2.1. through 3.2.7., as well as table 3.2.11a. and Fig. 3.2.11. in this section, the total increase of 2334 μmoles of the 22 amino acids in the skeletal muscle was found to be the greatest Among all the body fluids and tissue compartments examined, The percentage increases of the total amino acids in the FHF muscle, brain, kidneys and liver were 37%, 27%, 21% and 6% respectively.

Table 3.2.11b. displays the FHF/Normal ratios of the amino acids in various organs and tissues. From that table, it can be seen that of the 22 amino acids analyzed, 16 were significantly increased in the skeletal muscle, 7 were increased in the liver, 13 were increased in the brain, and 10 were increased in the kidneys. A total of 13 amino acids were significantly increased by more than 2 fold in the skeletal muscle tissue pool, whereas only 2 in the liver, 6 in the brain and only 1 in the kidney were found to have increased by more than 2 fold. The number of the amino acids showing more than 3 fold increase in the tissues were 4 in the skeletal muscle, and only 1 in each of the other three organs, liver, brain and kidneys.

Considering its total mass, the skeletal muscle is by far the biggest pool of free amino acids in the rats (Fig. 3.2.11.). The total weight of the skeletal muscle has been reported to be 45.5% of the body weight (Caster et al, 1956). The liver and the kidneys account for only 4.15% and 0.76% of the body weight respectively. The brain weighs only 0.55% of the total body weight. Bearing these figures in mind, further calculations of the total amount of the increased amino acids in the individual tissues would give very persuasive indication that the skeletal muscle is the biggest producer of the amino acid surplus in this GalN-FHF animal model. As can be seen from Fig. 3.2.11. and Table 3.2.11a., when the total amino acids in the FHF tissues were compared with those in the normal tissues, the net increase of 2334.2 μmoles in the total muscle mass was 50 times greater than that in the total liver tissue, 40 times that the kidneys, 120 times that the brain tissue, and 11 times that of the plasma.

The blood plasma has a relatively smaller pool size in comparison to the other tissues. Plasma concentration of total amino acids (expressed as μmol/ml) was less than 10% of the muscle, the liver, and the brain, and only 3% of the kidneys (expressed as μmol/g). Amino acid concentrations in the total blood are very close to the plasma levels with only a slight discrepancy in a few amino acids. The plasma volume is about 3.4% of the total body weight. The total muscle mass is more than 13 times that of the total plasma volume size. Since in normal rats the amino acid concentration in the muscle is more

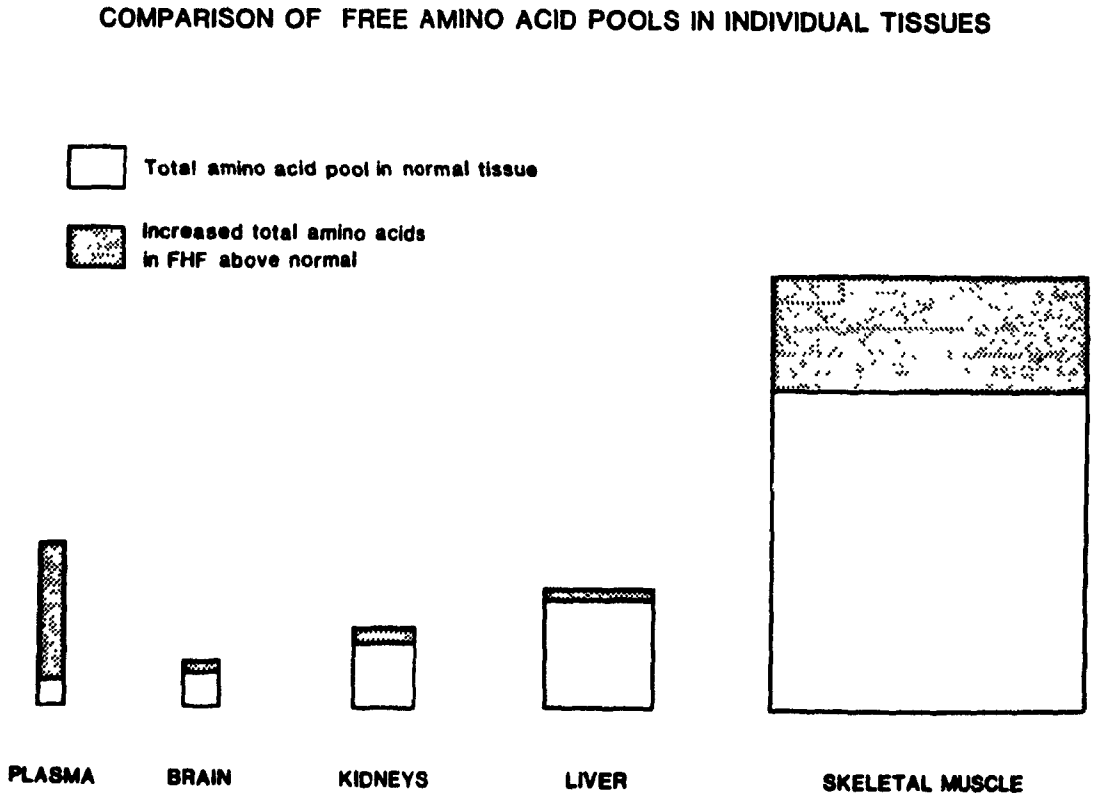


Fig. 3.2.11. Total amino acid pools are calculated and expressed as the area of the squares in plasma, brain, kidney, liver and skeletal muscle tissues. The individual values of the amino acid pools are shown in Table 3.2.11a. (p 148).

TABLE 3.2.11a.

TOTAL FREE AMINO ACIDS IN INDIVIDUAL TISSUE POOLS

Tissue	Mass (g)	NORMAL AA TOTAL		FHF AA TOTAL		AA IN POOLS	
		Concen-tration (mol/g)	Total pool (moles)	Concen-tration (mol/g)	Total pool (moles)	Total Increase (%)	Total Increase (mole)
Muscle	136.5	46.4	6333.6	63.5	8667.8	36.9%	2334.2
Liver	12.5	59.4	742.5	62.9	786.3	5.9%	43.8
Brain	1.7	43.5	71.8	55.2	91.1	26.9%	19.3
Kidneys	2.2	114.7	252.3	139.0	305.8	21.2%	53.5
Plasma	10.2	3.5	35.2	23.5	239.7	581.0%	204.5

Table 3.2.11b. FHF/normal ratios of amino acid concentrations in differing tissues and compartments

Amino Acid	Plasma	Portal Plasma	CSF	Liver	Muscle	Cerebral Hemisphere	Kidneys
ASP	3.19	4.60		1.36	1.67*	1.16	1.02
GLU	2.29*	2.96	0.28	1.50*	0.53	0.97	1.24*
ASN	7.90*	7.99*	2.61	1.27	2.46*	1.14	0.97
SER	5.82*	6.53*	1.66*	1.10	2.07*	1.13	1.12
HIS/GLN	7.61*	7.04*	2.65*	0.99	1.66	2.02*	1.57*
GLY	5.88*	5.86*	1.57	0.91	1.19	1.24*	1.38*
THR	9.90*	11.18*	3.70*	1.27*	3.58*	2.53*	1.46*
ARG	0.02*		0.40*			0.93	0.90
ALA	11.51*	11.01*	2.36*	1.43*	1.64*	1.69*	1.65*
GABA				2.14*		1.09	1.73
TAU	3.51*	3.57*	0.95	0.63	1.02	0.97	0.64*
TYR	23.32*	23.47*	16.76*	1.85*	6.70*	5.99*	1.62*
α-ABA				11.41*	14.54*		47.34*
VAL	3.38*	4.24*	3.51*	1.01	2.61*	1.78*	1.14
MET	9.07*	9.41*	5.76*	1.15	3.70*	2.58*	1.22
TRP	2.55*	2.96*	7.04*	1.62*	3.83*	2.46*	1.54*
ILE	2.67*	3.34*	2.43*	0.94	2.25*	1.39*	1.13
PHE	3.88*	4.77*	4.38*	0.98	2.25*	1.53*	1.14
LEU	3.37*	4.66*	3.67*	0.88	2.26*	1.44*	1.12
ORN	21.85*	20.82*	1.72	1.00	2.84*	1.41	1.80*
LYS	10.41*	11.07*	3.23*	1.57	2.06*	2.80*	1.61
TOTAL	6.65*	7.11*	2.62*	1.06	1.37*	1.27*	1.21

(1) All numbers with "*" symbol indicate the significant changes of amino acids in portal plasma, CSF, brain, liver, muscle and kidneys.

(1) Arginine was the only amino acid that decreased in plasma and CSF in FHF rats. Arginine was not detected in both normal and FHF livers.

(3) GABA was not detected in plasma, Portal plasma, CSF and skeletal muscle in both normal and FHF rats. The FHF/normal ratios were therefore not calculated.

(4) α-Amino-butyric acid was detected in FHF plasma, portal plasma, CSF, brain hemisphere, and both normal and FHF liver, muscle and kidneys, but no in normal plasma, portal plasma, CSF and brain hemisphere. The FHF/normal ratios were therefore only calculated for liver, Muscle, and kidneys

than 13 times higher than that in the blood plasma, the calculated total amino acid pool size in the mucsulature (6333.6 mol) is 174.2 times (13.4 X 13) as great as that in the plasma pool (35.7 μmol). In FHF, the total amino acid pool in the muscle (8667.8 μmol) is 36 times greater than that in the plasma pool (239.7 μmol) (Fig. 3.2.11. and Table 3.2.11a.).

Amino acid changes in the brain and in the kidney were similar. The numbers of increased amino acids were 13 in the brain and 10 in the kidneys. The major differences between the two organs were: (1) the 6 fold increase in the brain tyrosine, but only 1.6 fold in the kidneys, and (2) the 47 fold increase in α-ABA in the kidneys but undetectable in the brain. The other amino acids showed similar changes in both the brain and the kidneys in the FHF cats.

In the liver, however, most of the amino acids that increased did so by only 1.27 to 1.85 fold. The higher increments found in GABA and α-ABA were 2.1 and 11.4 respectively. The number of the elevated amino acids were only 7 in the liver as against 16 in the skeletal muscles.

The normal kidneys do not play a major role in the regulation of the free amino acids in the systemic circulation whether in the presence or absence of the liver (Flock et al, 1952). The concentrations of plasma free amino acids were normal in the dogs after nephrectomy, but were elevated in the dogs after combined nephrectomy and hepatectomy.

The amino acids in the CSF account for only a very small percentage in the total amino acid pool of the body. The total

amino acid concentration in CSF is less than 1/3 of that in plasma, and less than 1/40 of that in the brain.

3.2.12. Ratios of Amino Acids between Plasma and Various Tissues

Table 3.2.12. shows the ratios of various tissues or CSF to the plasma in amino acid concentrations in normal and FHF animals. The objective of this table is to further examine the relative rate of increase of respective amino acids in portal plasma, CSF, brain, liver, muscle and kidneys in FHF with reference to the rate of increase in the plasma. Thus, the normal tissue/plasma ratio of a particular amino acid was calculated with normal tissue concentration divided by normal plasma concentration, and the FHF tissue/plasma ratio was calculated in the similar way using tissue concentration divided by plasma concentration of each amino acid in FHF. The values of normal tissue/plasma ratio reflects the concentration difference which existed between this particular tissue and the plasma. Any increase in the ratio would indicate either an increase in the tissue or a decrease in the plasma, or both. Any decrease in the ratio would indicate either a decrease in the tissue or an increase in the plasma, or both. In FHF, the alterations in this ratio will mostly reflect the extent of amino acid increases since very few amino acids would decrease. The change of the ratio also suggests whether any correlative relationship exists in the two concerned tissues with regard to a given amino acid.

Due to an almost 7-fold increase in the concentration of

Table 3.2.12.
Tissue/plasma ratios of amino acid concentration

Amino acid	Portal plasma /Plasma		CSF /Plasma		Brain /Plasma		Liver /Plasma		Muscle /Plasma		Kidney /Plasma	
	Normal	FHF	Normal	FHF	Normsl	FHF	Normal	FHF	Normal	FHF	Normal	FHF
ASP	0.93	1.34	0.11		398	145	98.0	41.8	21.7	11.4*	472	151
GLU	1.26	1.62	0.11	0.01	84.7	35.9	32.6	21.3*	11.4	2.64*	114	61.7*
ASN	0.90	0.91*	0.07	0.02	2.86	0.41	18.8	3.01	4.46	1.39*	54.3	6.64
SER	0.82	0.92*	0.34	0.10*	7.53	1.46	15.4	2.90	4.58	1.63*	43.7	8.41
HIS/GLN	0.86	0.79*	0.98	0.34*	10.0	2.66*	12.6	1.65	8.14	1.78	5.19	1.07*
GLY	1.01	1.00*	0.03	0.01	9.84	2.07*	24.25	3.73	11.0	2.22	45.2	10.6*
THR	0.79	0.89*	0.32	0.12*	3.92	1.00*	13.5	1.74*	3.43	1.24*	35.5	5.25*
ARG	0.76	0.95*	0.24	5.83*	2.57	144.0					31.6	1705
ALA	1.00	0.95*	0.12	0.02*	2.81	0.41*	13.2	1.63*	15.3	2.17*	18.4	2.64
GABA												
TAU	1.07	1.09*	0.15	0.04	37.5	10.4	33.1	5.89	77.2	22.4	61.7	11.2*
TYR	0.85	0.86*	0.10	0.07*	2.40	0.62*	19.3	1.53*	4.37	1.25*	41.9	2.91*
α-ABA		0.96		0.05		0.70		1.37*		1.02*		6.61*
VAL	0.85	1.06*	0.02	0.02*	1.08	0.57*	11.0	3.27	1.67	1.29*	29.7	10.0
MET	0.87	0.90*	0.10	0.06*	2.34	0.67*	16.7	2.11	3.42	1.40*	44.3	5.93
TRP	0.86	1.00*	0.03	0.07*	0.74	0.72*	3.05	1.94*	0.91	1.37*	7.77	4.71*
ILE	0.78	0.97*	0.02	0.02*	1.49	0.78*	11.8	4.16	2.19	1.85*	33.6	14.2
PHE	0.79	0.97*	0.05	0.06*	2.32	0.91*	13.6	3.42	3.47	2.01*	33.6	9.89
LEU	0.78	1.08*	0.03	0.03*	1.91	0.82*	15.9	4.16	2.59	1.74*	44.9	14.9
ORN	1.02	0.97*	0.15	0.01	2.20	0.14	54.6	2.49	1.68	0.22*	34.5	2.84*
LYS	0.94	1.00*	0.24	0.08*	1.26	0.34*	8.89	1.34*	2.67	0.53*	21.0	3.24*
TOTAL	0.88	0.94*	0.29	0.11	12.3	2.35*	16.8	2.67	13.1	2.70*	32.5	5.91

Notes:

(1) All numbers with "*" symbol indicate the significant changes of amino acids in portal plasma, CSF, brain, liver, muscle and kidneys.

(2) GABA was not detected in both normal and FHF plasma, therefore the tissue/plasma ratios were not calculated.

(3) α-Amino-butyric acid was detected in FHF, but not in normal plasma using the present technique. The tissue/plasma ratios were therefore only calculated in FHF rats.

total amino acids in plasma, which is far beyond the magnitudes of increases observed in all other tissues, the tissue/plasma ratios in most FHF animals were significantly lower than those in normals. The only two exceptions were Arg and Trp. The CSF/plasma, brain/plasma and kidney/plasma ratios of Arg were all increased in FHF. These changes were due to the diminution of arginine in plasma and the augmentation of arginine in CSF and the tissues. The CSF/plasma and muscle/plasma ratios of tryptophan concentrations were also increased in FHF. The reason for this may be found in the relatively greater augmentation of tryptophan in CSF and muscle than in the plasma.

Since α-amino-butyric acid (α-ABA) did not appear in normal plasma, portal plasma, CSF, and brain, the ratio values were unattainable in the normal animals.

3.2.13. Changes of the BCAA/AAA Molar Ratio in FHF

The BCAA/AAA molar ratios in FHF plasma in this study decreased significantly from 3.07 to 0.78 on average (Table 3.2.13.). This was comparable to the findings of William's group of 3.54 $\pm$ 0.13 in normal controls, 1.00 $\pm$ 0.13 in hepatic coma surviving patients and 0.72 $\pm$ 0.13 in non-survivors (Chase et al, 1978). In this report of the BCAA/AAA molar ratio changes in portal and systemic plasma, CSF, brain, liver, muscle, and kidneys has provided a detailed insight of amino acid ratio changes in the various organs and tissues. The

BCAA/AAA ratio changes in the liver, the skeletal muscle and the kidneys in FHF have never before been documented. As far as the BCAA/AAA molar ratios are concerned, the amino acid derangements found in my study of FHF rat model are not in conflict with Fischer's false neurotransmitter theory.

Table 3.2.13.

BCAA/AAA MOLAR RATIOS IN VARIOUS TISSUES

IN NORMAL TNA FHF RATS

Tissues	Normal rats	FHF rats	Significance
Plasma	3.06 ± 0.24	0.80 ± 0.22	$p < 0.001$
Portal plasma	3.02 ± 0.28	1.00 ± 0.21	$p < 0.001$
CSF	1.03 ± 0.18	0.31 ± 0.10	$p < 0.001$
Brain	1.92 ± 0.06	0.85 ± 0.20	$p < 0.001$
Liver	2.46 ± 0.09	1.61 ± 0.26	$p < 0.001$
Kidney	2.90 ± 0.12	2.42 ± 0.23	$p < 0.002$
Muscle	1.68 ± 0.10	0.92 ± 0.19	$p < 0.001$

1. BCAA/AAA ratio denotes the molar ratio of the concentrations (umol/ml, or, umol/g) branched chain amino acids to aromatic amino acids. This molar ratio value is expressed as:

   BCAA / AAA = Val+Leu+Ile / Tyr+Phe

2. There are four animals included in the normal group and six included in the FHF group.

3. Statistical significance was examined with Student t-test.

3.3. EFFECTS OF HEMOPERFUSION ON AMINO ACID LEVELS IN PLASMA, CSF AND BRAIN

3.3.1. In Vitro Adsorption Spectrum of Amino Acids by CAC

Two grams of collodion coated petroleum charcoal beads (CAC) removed 99% of the aromatic amino acids (AAA) including Tyr, Trp and Phe from a mixture of 20 amino acids in 20 ml 50 μM amino acid solution (Fig.3.3.1a). In the meantime, 50% to 81% of the BCAAs valine, leucine and isoleucine were removed. The acidic and basic amino acids being polar molecules were generally poorly adsorbed. Arginine was an exception in that it was adsorbed at a relatively greater percentage as compared with other acidic and basic amino acids.

As the concentration of the amino acids in solution was doubled from 50 μM to 100 μM the adsorption for BCAAs was approximately halved while the adsorption for AAAs still remained as high as around 90% (Fig. 3.3.1b). The adsorption spectrum for the whole range of amino acids exhibited the similar pattern as in the 50 μM solution but the magnitudes of adsorption were all reduced accordingly.

Amino acids generally fall into 3 chemical categories: neutral, acidic and basic. Acidic and basic amino acids are generally polar and hydrophilic molecules. These amino acids usually have a high solubility in aqueous solution and hence have a low affinity for the electrically neutral surface of the activated charcoal. Apart from the acidic and the basic amino acids, all the other amino acids are classified as neutral

amino acids and are relatively less polar and more hydrophobic than the other two groups. Among the neutral amino acids, those of importance in hepatic failure research are the aromatic amino acids which include Tyr, Phe and Trp; and the branched chain aliphatic amino acids which include valine, leucine and isoleucine.

Judging from the patterns of adsorption spectrum of the 20 amino acids displayed in Figures 3.3.1a and 3.3.1b, the removal of the molecules by collodion-coated activated charcoal (CAC) appeared to be roughly according to the order of the hydrophobicity. The amino acids with aromatic rings (AAA) apparently have the highest hydrophobicity and, therefore, the highest removal rate by charcoal. The branched-chain amino acids (BCAA) were adsorbed to a lesser extent than the AAAs. The removal of other neutral amino aicds by adsorption were even less than that of BCAAs, yet still greater than that of the basic amino acids, ie., Lys and Arg. There was virtually no adsorption for Asp and Glu, the acidic amino acids, under the conditions tested in this study. These two acidic amino acids definitely have the highest molecular polarity, and the lowest hydrophobicity among the amino acids studied here.

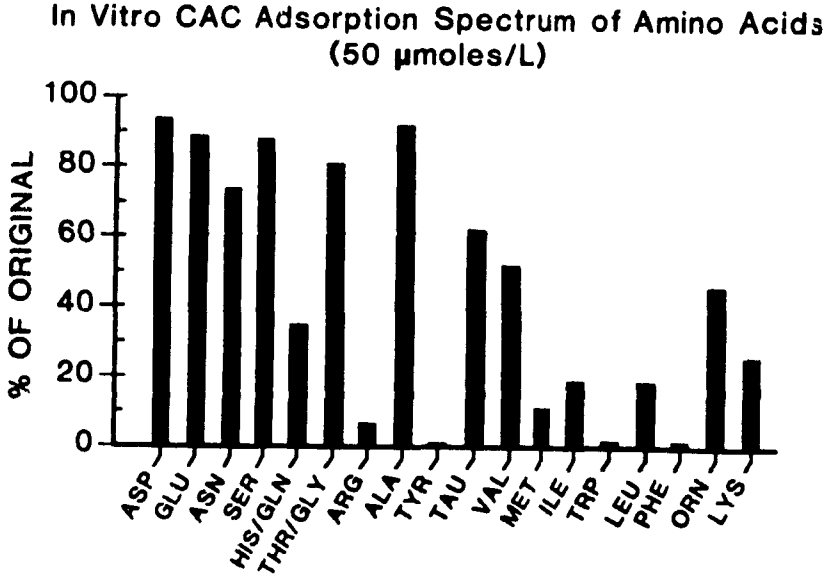


Fig. 3.3.1a. In vitro adsorption spectrum of amino acids by coated activated charcoal (CAC). There were 20 amino acids included in the 20 ml solution (50 µM). Two gramsof CAC were used for the adsorption test. Each column represents the amount of amino acid remained after the in vitro adsorption test.

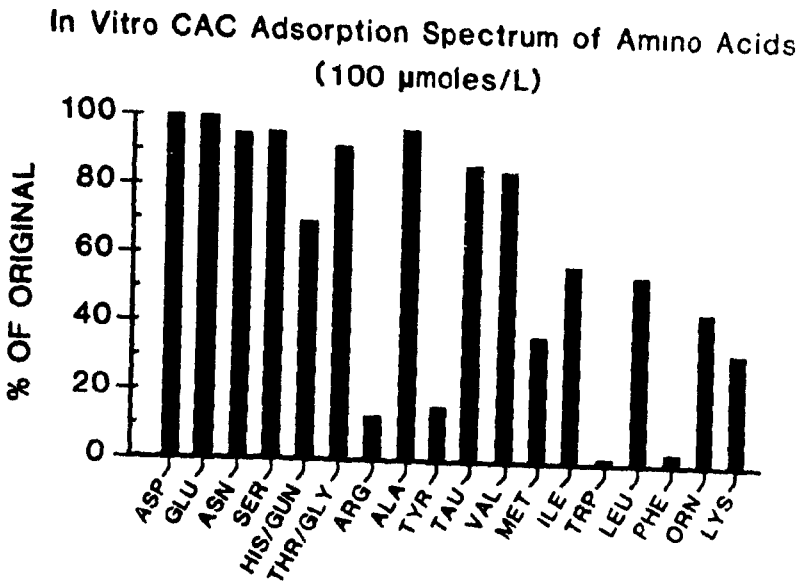


Fig.3.3.1b. In vitro adsorption experiment using 100 µM amino acid solution. The other experimental conditions were identical to the experiment using 50 µM amino acid solution.

3.3.2. Amino Acid Removal by in Vitro CAC Hemoperfusion

The removal of amino acids by collodion coated activated charcoal (CAC) was further studied in the in vitro hemoperfusion system using FHF rat blood. The in vitro hemoperfusion experiment displayed a spectrum of adsorption similar to that of the in vitro adsorption experiment using aqueous solution (Fig. 3.3.2a.). The total removal rates of AAAs, which ranged from 85 to 95% of the original, were still the most prominent. BCAAs were removed to a lesser extent than AAAs, but their removal was still greater than that of the other amino acids. Acidic and basic amino acids were adsorbed the least. Fig. 3.3.2b. shows that the clearance for AAAs was high in the first 30 minutes, then became a bit lower at 60 and 90 minutes. It was shown that after 85% to 95% of the total AAAs were removed by 60 minute hemoperfusion, continuing hemoperfusion to the 90 minute point offered further, although less significant, reductions of AAAs. A slight rebound of BCAA concentrations was revealed at 90 minutes of hemoperfusion while AAAs were still being removed (Fig. 3.3.2c.). This finding indicated a preferentially high affinity of activated charcoal to AAAs, resulting in the partial displacement of BCAA molecules by AAAs.

The concentration changes in AAAs and BCAAs during the in vitro hemoperfusion are listed in Table 3.3.2.. The molar ratio of BCAA/AAA was greatly improved from 1.09 at the begining to 3.87 at the end of hemoperfusion. This finding is

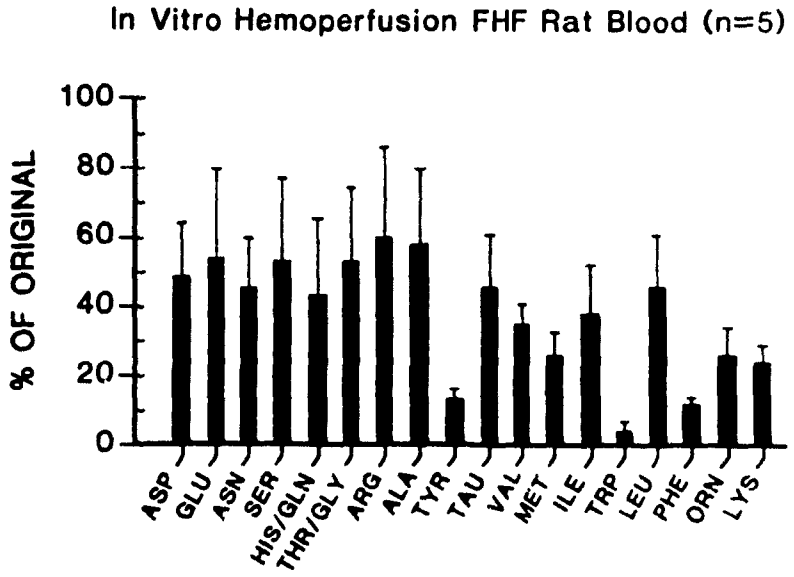


Fig. 3.3.2a. The removal of plasma amino acids after 90 min in vitro hemoperfusion. Five batches of 24 ml blood from GalN-induced FHF rats were used. Each batch of blood was perfused with 1.5 g CAC beads.

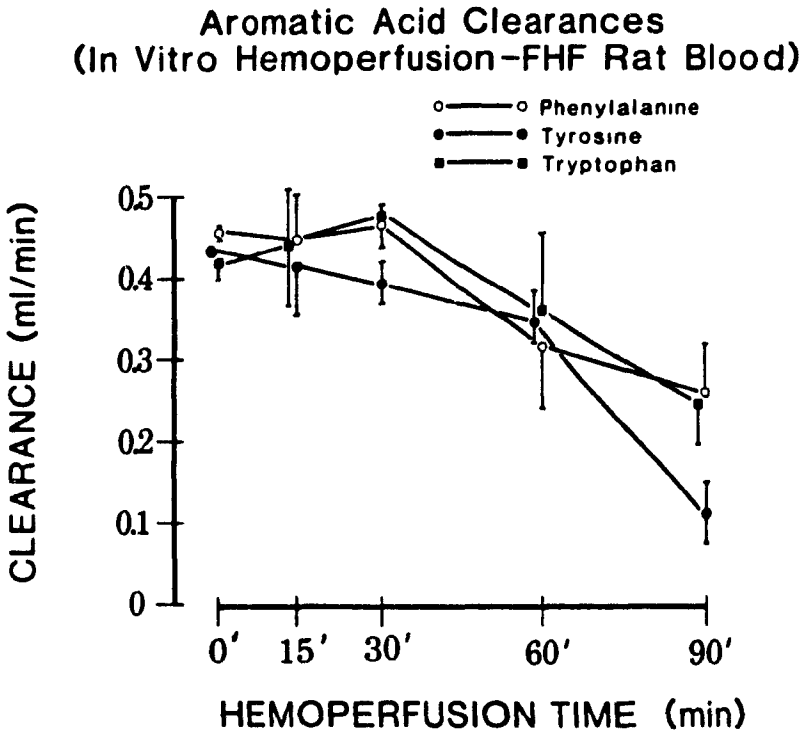


Fig. 3.3.2b. Clearance rates of plasma aromatic amino acids by in vitro hemoperfusion with collodion coated activated charcoal (CAC). Flow rate was 1.0 ml/min. Five batches of 24 ml blood from GalN-induced FHF rats were used in the hemoperfusion.

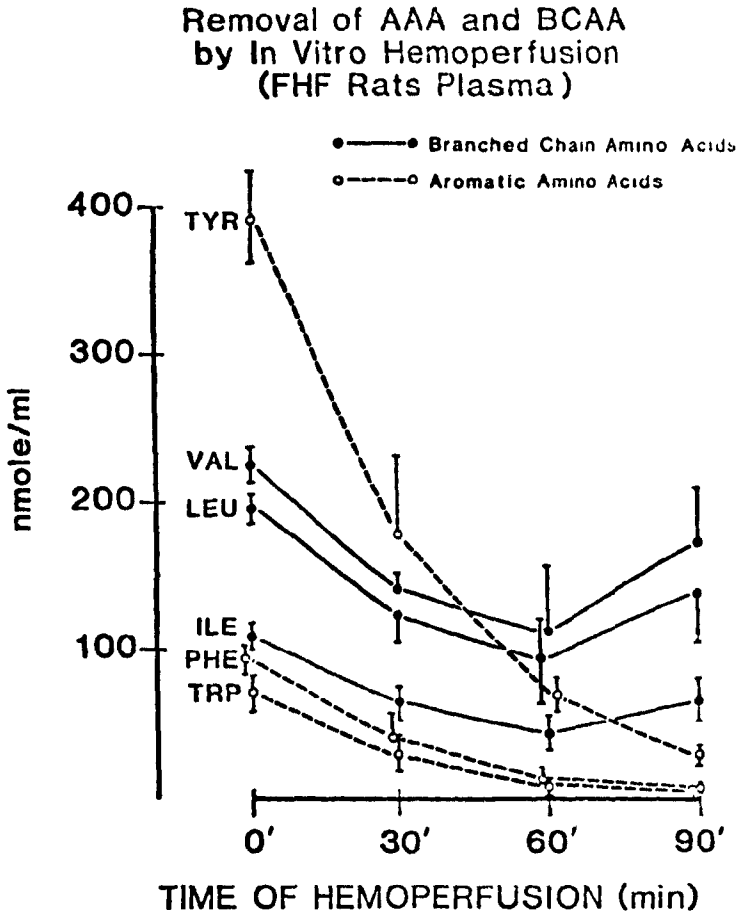


Fig. 3.3.2c. The concentrations of aromatic amino acids (AAA) and branched chain amino acids (BCAA) before, during and after 90 min in vitro hemoperfusion. The amino acid levels are tabulated in Table 3.3.2..

Table 3.3.2.

REMOVAL OF AROMATIC AND BRANCHED CHAIN AMINO ACIDS BY IN VITRO HEMOPERFUSION WITH MIXED RAT BLOOD (umolar)

	Time of Hemoperfusion (min)		
	0'	30'	60'
Tyrosine	393.0 ± 35.2	171.4 ± 57.3	51.9 ± 10.8
Phenylalanine	93.8 ± 6.8	40.4 ± 17.2	11.2 ± 2.0
Valine	223.7 ± 9.0	142.6 ± 10.2	111.6 ± 44.7
Isoleucine	108.8 ± 2.4	63.6 ± 10.3	41.4 ± 15.7
Leucine	197.1 ± 5.8	124.2 ± 15.4	91.4 ± 30.9
Molar $\frac{BCAA}{AAA}$ ($\frac{Val + Ile + Leu}{Tyr + Phe}$)	1.09	1.56	3.87

very encouraging as this type of improvement for the BCAA/AAA molar ratio has been proposed to be important in the reversal of hepatic encephalophthy (Rossi-Fanelli et al, 1982).

3.3.3. Effects of 1 Hour CAC Hemoperfusion on Plasma and CSF Amino Acid Levels

Ten GalN-FHF rats in grade III hepatic coma were studied in this experiment. Rats were randomly divided into 2 groups: 5 rats in the CAC hemoperfusion group and 5 in the control hemoperfusion group.

Amino acid levels in plasma samples before hemoperfusion from both groups were considered as the control baseline levels. These control baseline levels were then compared with plasma amino acid levels after 1-hour collodion coated charcaol hemoperfusion or after 1-hour control hemoperfusion. The statistical analysis was performed using the Student t-test. There was no significant difference between baseline levels and the levels after control hemoperfusion. However, after 1-hour charcoal hemoperfusion, plasma levels of Tyr, Phe and Trp were all significantly reduced when compared with control baseline levels (Table 3.3.3a.). The BCAAs Val, Ile and Leu were also lowered significantly but not as much as AAAs. The magnitudes of removal of the amino acids by activated charcoal adsorption were, by and large, in a similar pattern as that seen in the in vitro experiments. The BCAA/AAA molar ratio was somewhat improved although a statistical significance was not observed.

Despite the changes in the plasma amino acid profile, CSF

EFFECT OF 60 MIN. HEMOPERFUSION
ON PLASMA AMINO ACID LEVELS
(nMole/ml, n=15)

	Control Hemoperfusion	Charcoal Hemoperfusion	Student 't' test
ASP	33 35±4 66	21 21±4 73	NS
GLU	232 42±43 58	149 78±30 00	P<0 01
ASN	258 73±52 45	220 43±30 12	NS
SER	804 09±174 96	689 20±59 95	NS
HIS/GLU	3419 72±886 66	3030 3 ±317 76	NS
THR/GLY	1791 08±343 80	1592 32±191 62	NS
ARG*	non detectable		NS
ALA	3712 71±823 58	3171 66±668 55	NS
TYR	1085 35±338 72	696 16±214 65	P<0 05
TAU	453 74±176 37	640 40±264 68	NS
VAL	463 87±50 29	396 80±23 67	P<0 05
MET	315 04±91 01	238 46±29 61	NS
ILE	203 85±26 90	164 10±19 20	P<0 05
TRP	153 61±26 04	104 17±10 55	P<0 01
LEU	398 73±54 51	329 07±28 15	P<0 05
PHE	178 13±38 77	132 92±10 53	P<0 01
ORN	598 53±107 19	522 55±126 90	NS
LYS	2061 41±390 66	1565 13±432 38	P<0 01
Ratio $\frac{VAL+ILE+LEU}{TYR+PHE}$	0 84±0 293	1 07±0 237	NS

Table 3.3.3a. Plasma amino acid levels after 1-hr CAC or control hemoperfusion. All values are the means ± SD. The Val+Ile+Leu/Tyr+Phe molar ratio (BCAA/AAA) was not significantly different between the two groups.

EFFECT OF 60 MIN. HEMOPERFUSION ON CSF AMINO ACID LEVELS

(nMole/ml)

	Control (n=5) Hemoperfusion	ACAC (n=5) Hemoperfusion	Student 't' test
ASP			NS
GLU	3 13±1 26	5 90±6 76	
ASN	7 10±1 49	11 0 ±9 87	
SER	91.38±15.91	90 71±19 45	
HIS/GLU	772.62 ±111 69	864 00±152 41	
THR/GLY	70 63±12 74	68 26±8 53	
ARG	17 15±7 79	13.04 ±3 06	
ALA	95 34±34 81	99 79 ±24 32	
TYR	83 44±23 18	76 73 ±31 01	
TAU	19 28±3 84	21 08±2 53	
VAL	9 91±1 44	11 03±2 79	
MET	20 57±4 62	20 04 ±3 28	
ILE	4.20±0 98	3.82 ±1 65	
TRP	6 80±1 20	6 16 ±1 59	
LEU	13.23 ±2 22	17 34 ±7 03	
PHE	13 86 ±1 96	12 47 ±2 09	
ORN	10.59 ±3 53	8 10±2 19	NS
LYS	152 21 ±22 54	164 59 ±22 59	
Ratio $\frac{VAL+ILE+LEU}{TYR+PHE}$	0 28 ± 0 06	0 38±0 14	NS

Fig. 3.3.3b. CSF amino acid concentrations in CAC and control hemoperfusion groups. No significant reduction of any amino acid was found.

amino acid concentrations seemed unaffected by activated charcoal hemoperfusion (Table 3.3.3b.). The BCAA/AAA molar ratio was 0.38 ± 0.14 in the CAC hemoperfusion group, and 0.28 ± 0.06 in the control group. There was no significant difference between the two groups.

3.3.4. Effects of 2 Consecutive 1-hour CAC Hemoperfusion on Plasma, CSF and Brain Amino Acids

In this experiment, plasma amino acid concentrations before and after hemoperfusion were compared and examined by the paired t-test in each of the two groups, ie., charcoal hemoperfusion group or control hemoperfusion group. When rats received 2 consecutive 1-hour hemoperfusions using activated charcoal, the pattern of reduction of most amino aicds in plasma was similar to that found in single 1-hour hemoperfusions (Table 3.3.4a.). All AAAs (Tyr, Trp and Phe) were significantly decreased. Among BCAAs, Val and Ile concentrations were significantly reduced. There was a 17% decrease on average in plasma Leu concentration after charcoal hemoperfusion treatment, but it was not statistically significant. Methionine was also substantially decreased in the charcoal-treated group. The values of BCAA/AAA molar ratio in plasma had increased from 0.70 ± 0.18 to 0.82 ± 0.22, however the difference was not statistically significant.

In the control experiment, i.e., 2 consecutive 1-hour hemoperfusions using empty columns, no statistical difference was seen between the plasma amino acid levels before and after

PLASMA AMINO ACID LEVELS BEFORE AND AFTER 2 HOUR CAC HEMOPERFUSION IN FHF RATS

(nMole/ml, Mean±SD)

	Before HP (n=4)	After HP (n=4)	Paired 't' test
ASP	39 38±12 19	28 28±15 20	NS
GLU	231.52±46 10	177 75 ±53 24	NS
ASN	384 79±44 15	377 17 ±72 18	NS
SER	1265 45±76 05	1193 26± 96 75	NS
HIS/GLU	4135 66±294 04	4137 45±445 95	NS
THR/GLY	2443 55±162 81	2519 08±177 12	NS
ARG	18 64±11 76	24 11±19 67	NS
ALA	3802 76±48 74	3950 18±163 50	NS
TYR	1592 11±77 23	1148 53±235 90	P<0 01
TAU	391 62±126 54	533 28±204 50	NS
VAL	605 56±86 69	514 53±44 99	P<0 05
MET	618 91±273 18	472 05±249 91	P<0 01
ILE	259 01±50 22	204 98 ± 21 54	P<0 05
TRP	280 71±22 45	156 30 ± 38 33	P<0 01
LEU	514 26±106 46	422 92±38 09	NS
PHE	426 42±227 54	345 68±198 56	P<0 01
ORN	1054 42±77 95	923 93±139 46	NS
LYS	2365 35±43 59	2865 47±302 49	NS
VAL+ILE+LEU / TYR+PHE	0 6995±0 1796	0 8188±0 2244	NS

Fig. 3.3.4a. Plasma amino acid concentrations before and after 2 consecutive 1-hr CAC hemoperfusion in FHF rats with grade 3 coma. The concentration of total plasma aromatic acids were reduced by 28% after hemoperfusion as compared to 17% for the total plasma BCAA.

PLASMA AMINO ACID LEVELS BEFORE AND AFTER
2 HOUR CONTROL HEMOPERFUSION IN FHF RATS

(nMole/ml, Mean±SD)

	Before HP (n=4)	After HP (n=4)	Paired 't' test
ASP	53.29±25 80	62 30±52 59	NS
GLU	230 46±56 58	357 03±313 03	
ASN	373.90±73 33	396 49±128 24	
SER	1386 63±218 58	1379 37±191 85	
HIS/GLU	3999.70±485 92	4140.17±859 52	
THR/GLY	2598.14±344.18	2548 69±555 27	
ARG	11 04±9 73	22 94±31 73	
ALA	3777.99±531.05	3890 78±685 43	
TYR	1619 60±383.61	1483 28±355.10	
TAU	323.05±80 39	548 96±362 99	
VAL	574 61±189.45	574 15±175.82	
MET	515.52±59.36	505.41±52 19	
ILE	241.24±77 75	250 29±85 02	
TRP	286 34±69 39	255 52±75 69	
LEU	488.45±173 36	510 92±177 35	
PHE	342.21±27 49	374 76±73 25	
ORN	1030.41±232 54	1042 14±214 90	
LYS	3060 44±671 71	3233 18±642 41	NS
$\frac{VAL+ILE+LEU}{TYR+PHE}$	0 6624±0 1707	0 7064±0 1000	NS

Fig. 3.3.4b. Plasma amino acid concentrations were not reduced by the 2-hour control hemoperfusion.

CSF AMINO ACID LEVELS IN FHF RATS
AFTER ACAC OR CONTROL HEMOPERFUSION

	Control HP nMole/ml, M±SD	ACAC HP nMole/ml, M±SD	Paired 't' test
ASP	1 34±1 01	0.57±0 20	NS
GLU	6 11±5.24	1.71±0 11	NS
ASN	13 11±4 66	7 38±0 93	NS
SER	122.64±24.07	89 02±8 18	NS
HIS/GLU	1042.75±121 36	965.99±75.60	NS
THR/GLY	180.04±45.59	121 94±23.04	NS
ARG	19.21±5 54	16.92±3 38	NS
ALA	150.93±63 07	105 98±39 83	NS
TYR	151.17±59 30	94 95±37.66	P<0.05
TAU	27.94±11.19	16 02±2 70	NS
VAL	14.73±9.52	11.79±3 43	NS
MET	38.64±1.46	31.91±1 88	NS
ILE	6.84±2.76	4.29±1.82	NS
TRP	10.41±2 60	8.13±3.24	P<0.05
LEU	20.32±6.20	15.13±4.94	NS
PHE	30.12±14 71	23 86±15.77	P<0.05
ORN	19.44±12.27	11 68 ±5 54	NS
LYS	251.93±58.86	179.44±24 10	NS
$\frac{\text{VAL+ILE+LEU}}{\text{TYR+PHE}}$	0 2682±0 0950	0 2904±0 1205	NS

Table 3.3.4c. CSF amino acid concentration changes after collodion coated activated charcoal (CAC) or control hemoperfusion. Aromatic amino acids Tyr, Trp and Phe concentrations were significantly decreased in CAC hemoperfusion treated group.

BRAIN HEMISPHERE AMINO ACID LEVELS IN FHF RATS
AFTER CAC OR CONTROL HEMOPERFUSION

(nmole/g wet tissue, Mean ± SD)

Amino Acids	Control	CAC	Significance
ASP	3239.0 ± 776.9	2937.1 ± 879.7	N.S.
GLU	5396.3 ± 596.6	4624.0 ±1481.6	N.S.
ASN	97.8 ± 25.7	93.3 ± 18.6	N.S.
SER	1442.1 ± 394.3	1140.9 ± 305.5	N.S.
HIS/GLN	9058.1 ± 864.2	8250.3 ± 872.7	N.S.
THR/GLY	3235.0 ± 885.8	2565.9 ± 690.9	N.S.
ARG	446.0 ± 91.9	455.1 ± 121.0	N.S.
ALA	1773.5 ± 666.9	1815.7 ± 643.4	N.S.
TYR	846.3 ± 243.3	621.1 ± 219.7	N.S.
TAU	6431.0 ± 867.7	5716.3 ±1251.1	N.S.
VAL	239.9 ± 93.5	210.5 ± 69.6	N.S.
MET	270.9 ± 87.2	237.1 ± 69.5	N.S.
ILE	200.9 ± 40.0	162.0 ± 34.2	N.S.
TRP	102.4 ± 25.1	89.5 ± 22.1	N.S.
LEU	344.8 ± 103.2	320.7 ± 96.6	N.S.
PHE	273.6 ± 53.4	239.4 ± 58.4	N.S.
ORN	81.1 ± 46.7	47.0 ± 17.4	N.S.
LYS	900.8 ± 394.3	713.0 ± 144.1	N.S.
Ratio BCAA/AAA	0.73 ± 0.22	0.85 ± 0.25	N.S.

Table 3.3.4d. Amino acid levels in FHF brain after CAC or control hemoperfusion. No significant difference was found between the two groups.

the 2-hour procedures (Table 3.3.4b.).

In CSF, the concentrations of three AAAs Tyr, Phe and Trp were significantly lower in the hemoperfusion treated group than the control group (Table 3.3.4c.). Other neutral amino acids, including BCAAs in hemoperfusion treated group, also showed some reductions but with no statistical difference. The BCAA/AAA molar ratio was 0.27 ± 0.10 in the control group and 0.29 ± 0.12 in the hemoperfusion treated group. The two values were not significantly different. There was no significant decrease in any amino acid concentration in the brain of hemoperfused rats in comparison to the control subjects.

3.3.5. Effects of Tyrosinase Artificial Cell Hemoperfusion on Blood and Brain Tyrosine Levels

Hemoperfusion using tyrosinase artificial cells had a very high clearance of tyrosine from the circulation (Fig. 3.3.5a.). In the in vivo experiment, when hemoperfusion flow rate was 1.0 ml/min. the clearance was 0.42 ml/min. Even at 60 minutes after the onset of hemoperfusion, the clearance was still as high as 0.3 ml/min.

Such a high clearance brought a significant decrease in serum tyrosine level (Fig. 3.3.5b.) from an initial level of 1.15 ± 0.30 μM/ml to 0.47 ± 0.20 μM/ml at the end of 2 hrs hemoperfusion ($P<0.05$). When the tyrosine level in the experimental group after hemoperfusion was compared with the control group, the difference was also statistically significant (Fig. 3.3.5b.).

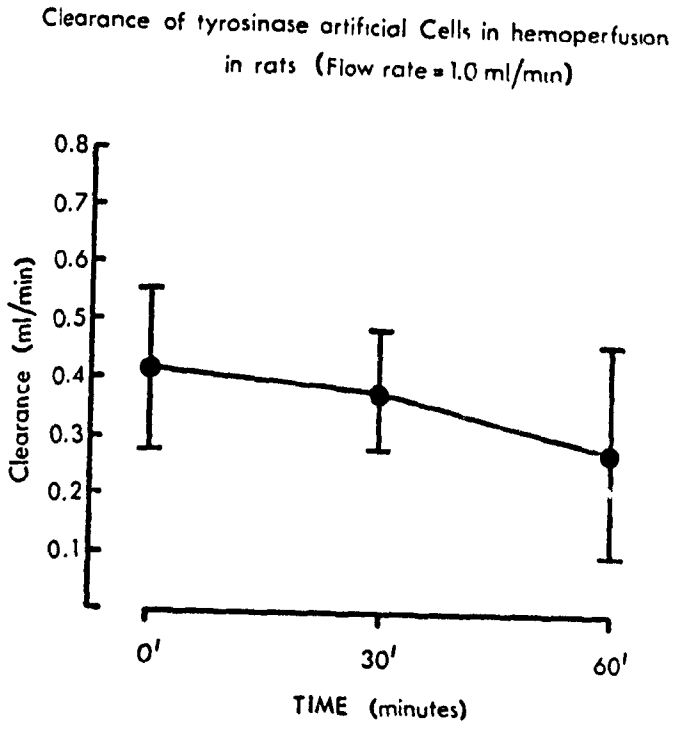


Fig. 3.3.5a. Tyrosine clearance rate in hemoperfusion using tyrosinase artificial cells. Flow rate was 1.0 ml/min. Tyrosine was measured with manual fluorometric method.

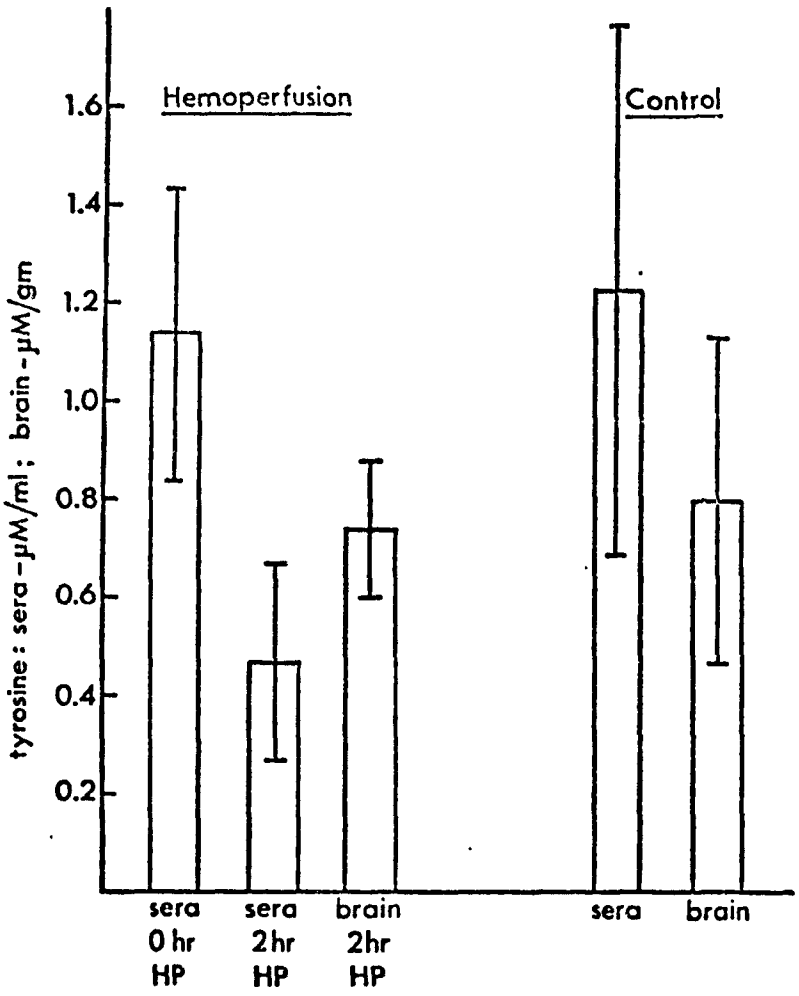


Fig. 3.3.5b. Tyrosine levels in serum and brain samples of FHF rats (grade 3 hepatic coma) treated with tyrosinase artificial cell hemoperfusion or control hemoperfusion. Two hour hemoperfusion using tyrosinase artificial cells resulted in a significant decrease in tyrosine concentration in the serum (from 1.15 $\pm$ 0.30 μmole/ml before hemoperfusion to 0.47 $\pm$ 0.20 μmole/ml after hemoperfusion. However, brain tyrosine level in the tyrosinase hemoperfusion group (0.74 μmole/g) was not significantly different from the control group (0.80 $\pm$ 0.33 μmole/g).

Although high clearance in hemoperfusion markedly reduced serum tyrosine concentration by more than half, the brain level of tyrosine was still unaffected (Fig. 3.3.5b.).

3.4. MIDDLE MOLECULE CHANGES IN BLOOD AND BRAIN COMPARTMENTS

3.4.1. Middle Molecules in FHF Rat Plasma

As shown in Fig. 2.4.1. (p.101 in chapter 2), the G-15 gel permeation column was calibrated with the standard chromatogram of the molecular weight markers. Blue dextran with a molecular weight of 2,000,000 is eluted at the void volume of the G-15 Sephadex gel column. Vitamin B12 is indicative of the middle molecule peak since its molecular weight (MW 1,355) falls within the proposed molecular weight range of the middle molecules (MW 500 - 2,000 or 1,000 - 1,500). The peaks of creatinine (MW 113) and uric acid (MW 168) indicate the elution volumes of the small solutes, such as salts, glucose and amino acids, etc..

In Sephadex G-15 gel permeation chromatography when monitored at 254 nm, peak 7 was found markedly elevated in FHF rat plasma (Fig. 3.4.1a.). In normal plasma samples, peak 7 was hardly discernible in the valley between peak 9 and peak 6 (Fig. 3.4.1a.). This result was in good accordance with the earlier studies carried out in this laboratory (Chang & Lister, 1980; Shi & Chang, 1982). The elution time of the elevated peak 7 coincided with Vit.B12 (MW: 1355) which was used as a marker for the middle molecule peak (Refer to Fig. 2.4.1.).

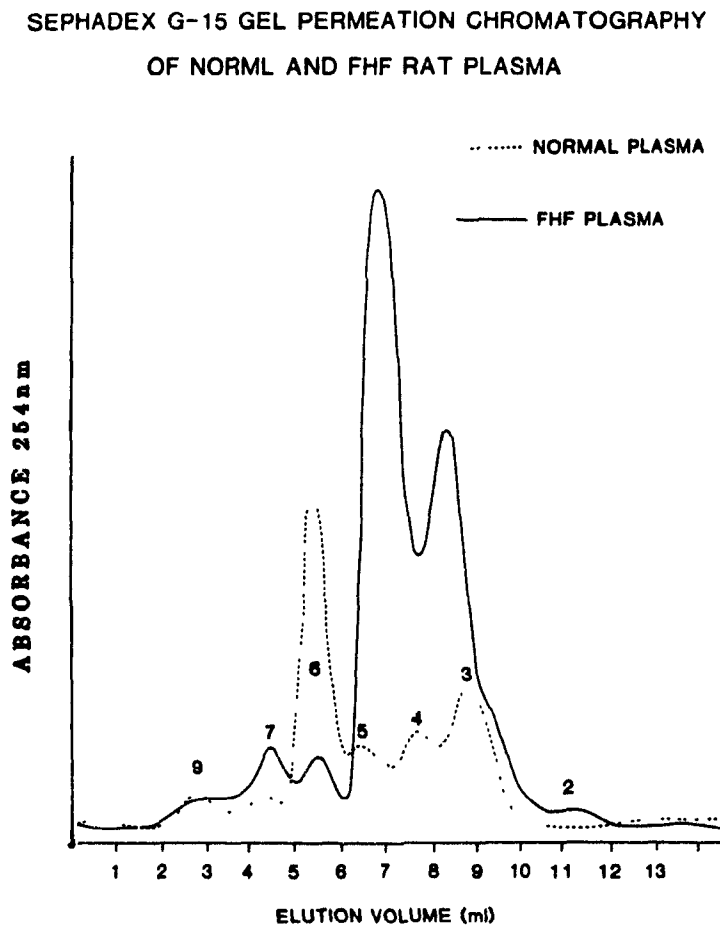


Fig.3.4.1a. Gel permeation chromatographies ofnormal and FHF rat plasma samples using Sephadex G-15 gels. Peak 9 is the peak at the void volume indicating macromolecules such as proteins. Peak 7 indicates the fraction of the middle molecules. The elution volume of peak 7 is identical to the peak of Vit.B12 shown in the standard chromatogram in Fig. 2.4.1.

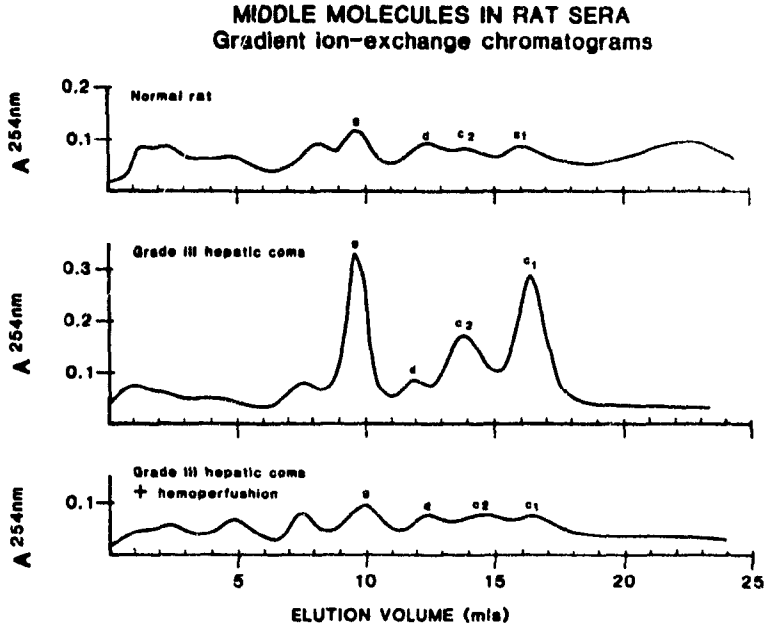


Fig. 3.4.1b. Ion-exchange chromatograms showing middle molecule subpeaks separated from peak 7 fractions. Peak 7 fractions were collected from normal, FHF control, and FHF hemoperfusion plasma samples. The subpeaks of peak 7 were named alphabetically in the reverse order of the elution time.

Top panel: Subpeaks from normal plasma peak 7.

Middle panel: Subpeaks from FHF plasma peak 7. The significant increase in subpeaks c1, c2 and g were shown.

Bottom panel: Subpeaks from plasma peak 7 fraction of hemoperfusion treated FHF rats. The decrease in subpeaks c1, c2, and g is shown.

Table 3.4.1.

MIDDLE MOLECULES IN RAT PLASMA

(PHU/ML)*

Group	$7c_1$	$7c_2$	7d	7g
Normal**	16.8 ± 8.8	12.1 ± 4.9	12.8 ± 3.1	28.4 ± 12.4
FHF***	25.7 ± 7.7	40.3 ±15.5	22.9 ± 9.4	45.3 ± 9.3
t-test	N.S.	P < 0.002	P < 0.05	P < 0.02

* Peak hight unit (PHU) is defined as 1/100 of a UV absorbance unit on spectrophotometric recordings. All values were expressed as mean ± 1 S.D..

** The normal group included 7 subjects.

*** The FHF group included 6 subjects.

When the fractions of peak 7 were further separated using gradient anion-exchange chromatography, several subpeaks were found elevated in FHF rat plasma compared to normal subjects. Subpeaks 7c2, 7d and 7g were significantly elevated. Subpeak 7c2 increased from a normal value of 12.1 ± 4.9 PHU/ml to 40.3 ± 15.5 PHU/ml. Subpeak 7d was 12.8 ± 3.1 in the normal while in the FHF plasma it was raised to 22.9 ± 9.4 PHU/ml. For subpeak 7g, the FHF value of 45.3 ± 9.3 was much higher than the normal value of 28.4 ± 12.4 PHU/ml. The magnitudes of elevation of these subpeaks from FHF plasma samples ranged from 1.6 to 3.3 folds when compared to the normal plasma samples (Fig. 3.4.1b. and Table 3.4.1.).

3.4.2. Middle Molecules in FHF Rat Brain

Sephadex G-15 gel permeation chromatography did not show any obvious differences in the elution peak patterns between normal and FHF rat brain samples. The peaks eluted at the void volume, at the middle molecular portion (peak 7 position), and at the bed volume, displayed almost identical profiles (Fig. 3.4.2a.).

In the gradient ion-exchange chromatography using DEAE Sephadex anion exchanger, however, there were significant increases of subpeaks 7d and 7g (Fig. 3.4.2b. and Table 3.4.2.). Subpeak 7d increased from 114.3 ± 62.0 PHU/g in normal rats to 207.1 ± 43.4 PHU/g in FHF subjects. Subpeak 7g elevated from a normal value of 203.4 ± 90.1 to 388.3 ± 158.4 PHU/g in FHF rats.

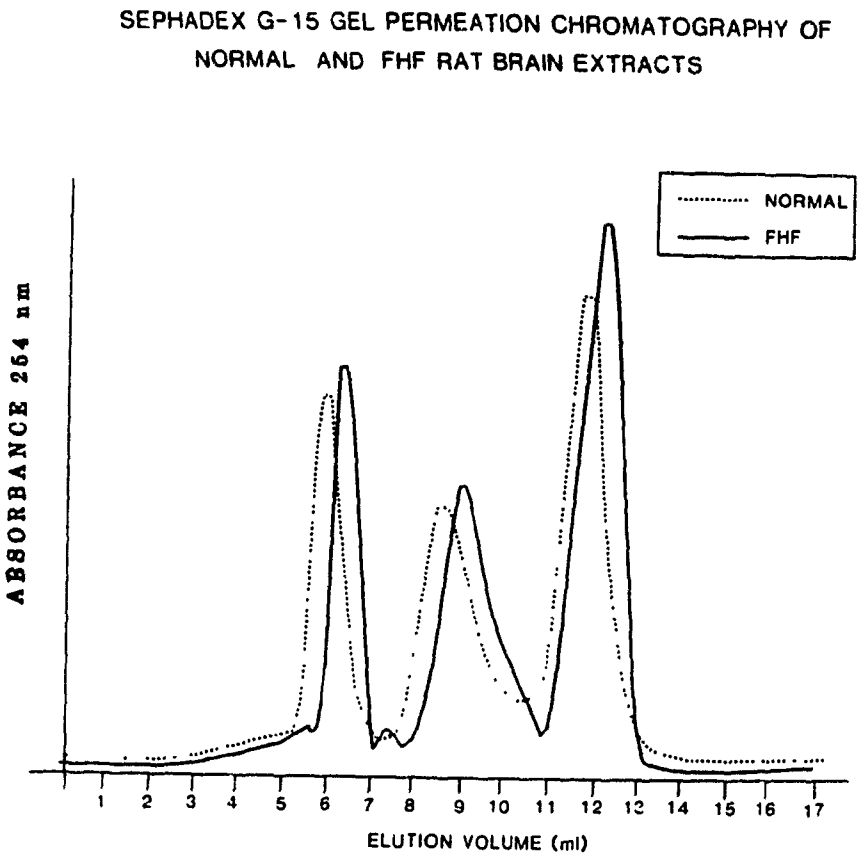


Fig. 3.4.2a. Sephadex G-15 gel permeation chromatographies of normal and FHF rat brain extracts. No significant change in the peaks is shown between the normal and FHF brain samples.

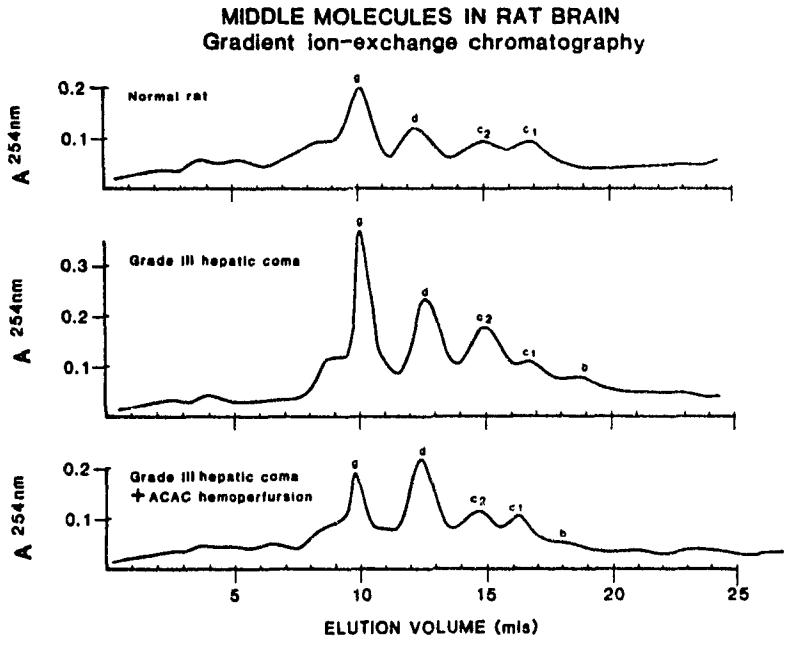


Fig. 3.4.2b. Ion-exchange chromatograms showing middle molecule subpeaks separated from peak 7 fractions from normal, control FHF, and hemoperfusion treated FHF rat brain samples.
Top panel: Normal rat brain.
Middle panel: Rat brain from FHF (grade 3 coma) rats.
Bottompanel:Rat brain from hemoperfusion treated FHF (grade 3 coma) rats

Table 3.4.2.

MIDDLE MOLECULES IN RAT BRAIN

(PHU/g)*

Group	$7c_1$	$7c_2$	7d	7g
Normal*	69.3 ± 23.6	87.3 ± 38.3	114.3 ± 62.0	203.4 ± 90.1
FHF**	90.3 ± 22.7	120.9± 34.9	207.1 ± 43.4	388.3 ±158.4
t-test	N.S.	N.S.	P < 0.05	P < 0.05

* PHU denotes Peak hight Unit. One PHU is equivalent to 1/100 of an absorbance unit on UV spectrometric recordings. All values were expressed as mean ± 1 S.D..

** The normal group included 4 animals.

*** The FHF group included 7 animals.

Table 3.4.3a.

REDUCTION OF PLASMA MIDDLE MOLECULES AFTER HEMOPERFUSION

(PHU/ml)*

Group	$7c_1$	$7c_2$	7d	7g
FHF**	25.7 ± 7.7	40.3 ±15.5	22.9 ± 9.4	45.3 ± 9.7
FHF+HP***	8.9 ± 1.3	10.6 ± 4.5	10.6 ± 1.4	11.6 ± 4.5
t-test	P < 0.01	P < 0.01	P < 0.02	P < 0.001

* PHU denotes Peak hight Unit. One PHU is equivalent to 1/100 of an absorbance unit on UV spectrometric recordings. All values were expressed as mean ± 1 S.D..

** FHF group included 6 subjects.

*** FHF+HP(hemoperfusion) group included 3 subjects. Hemoperfusion was performed using bead-type activated charcoal (Hemo France, France) for 1 hr.

Table 3.4.3b.

REDUCTION OF MIDDLE MOLECULES IN BRAIN CORTEX AFTER HEMOPERFUSION

(PHU/g)*

Group	$7c_1$	$7c_2$	7d	7g
FHF**	90.3 ±22.7	120.9±34.9	207.1±43.4	388.3±158.4
FHF+HP***	102.5±12.4	110.6±22.9	194.2±97.4	181.0±47.7
t-test	N.S.	N.S.	N.S.	P < 0.01

* PHU denotes Peak height Unit. One PHU is equivalent to 1/100 of an absorbance unit on UV spectrometric recordings. All values were expressed as mean ± 1 S.D..

** FHF group included 7 subjects.

*** FHF + HP (hemoperfusion) group included 5 subjects. Hemoperfusion was performed using bead-type activated charcoal (Hemo France, France) for 1 hr.

3.4.3. Reduction of Middle Molecules in FHF Rat Plasma and Brain after Hemoperfusion

The subpeaks 7d, 7c1, 7c2 and 7g of the plasma samples from the hemoperfusion treated rats showed very significant reductions when compared with nonperfused FHF subjects (Table 3.4.3a. and Fig. 3.4.1b.). Subpeak 7c1 in hemoperfused FHF rat plasma was decreased from 25.7 $\pm$ 7.7 to 8.9 $\pm$ 1.3 PHU/ml, subpeak 7c2 decreased from 40.3 $\pm$ 15.5 to 10.6 $\pm$ 4.5 PHU/ml, subpeak 7d decreased from 22.9 $\pm$ 9.4 to 10.6 $\pm$ 1.4 PHU/ml, and subpeak 7g decreased from 45.3 $\pm$ 9.7 to 11.6 $\pm$ 4.5 PHU/ml. All the reductions were examined with Student t-test and all were proven to be significant. The reductions of subpeaks 7g and 7c2 were the most prominent. In the brain, however, only subpeak 7g was found significantly lower in ACAC hemoperfused rats than nonperfused subjects (Table 3.4.3b. and Fig. 3.4.2b). The reduction magnitude of brain 7g was not as much as that of plasma 7g. In plasma, PHU values of subpeak 7g were reduced to 1/4 of the control value while in the brain it was halved. The other peaks 7c1, 7c2 and 7d displayed almost identical PHU values in both treated and untreated subjects. Therefore, these subpeaks were not affected by hemoperfusion using collodion coated activated charcoal (CAC).

3.4.4. Existence of Peptidic Substances in Middle Molecular Fractions from Liquid Chromatography

The Bio-Rad protein assay has revealed the existence of a very high content of protein-dye-positive substances within

peak 7g fraction of FHF plasma samples (Fig. 3.4.4a.). Considering the molecular size restriction of peak 7 fraction from G-15 gel permeation chromatography, the contents of 7g probably include peptide molecules. The protein dye-positive substances also existed in other subpeak fractions, but they were relatively much lower than that of 7g.

To exclude the possibility of amino acid interference in the subpeak 7g protein assay, the 7g fraction was run on the reverse-phase HPLC system. The HPLC reagents, eluents, equipments and techniques were all described in sections 2.2.3. and 2.2.4. of this thesis. The HPLC chromatogram displayed an almost straight baseline with no detectable amino acid peaks (Fig. 3.4.4b.). This further supported the peptide nature of some of the subpeak 7g contents.

3.4.5. Middle Molecular Peptides Determined by SDS-Polyacrylamide GEL Electrophoresis and the Estimation of Molecular Weight

When plasma samples from FHF rats were electrophoresed in Sodium Dodecyl Sulfate (SDS)-Polyacrylamide gel, a silver-stained peptide band was revealed (Fig. 3.4.5a.). Three samples of FHF grade III coma, and 1 out of 2 grade II coma samples displayed a silver-stained peptide band. This band was not detectable by Coomaasie Brilliant Blue R250. Silver stain is 10-50 fold more sensitive than Coomassie Brilliant Blue for proteins and peptides (Bio-Rad Laboratories, Bulletin 1089, 1982). Although the crude nature of the plasma sample created a noisy background, the peptide band was still clearly distinct

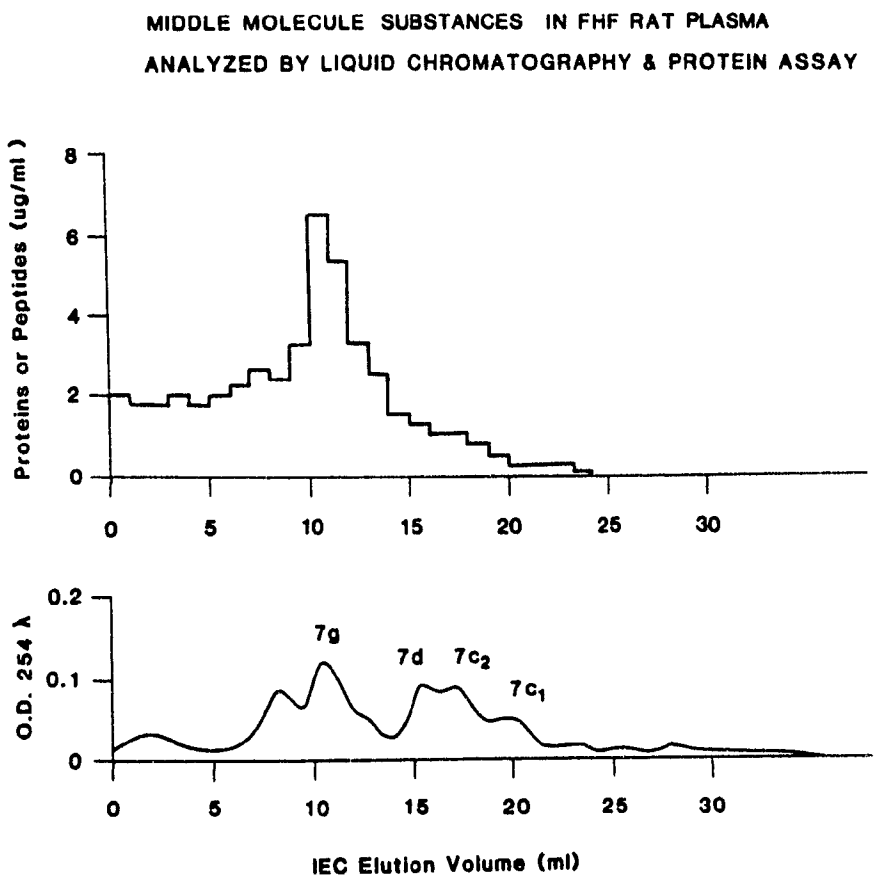


Fig. 3.4.4a. The determination of peptide substances in middle molecule subpeak fractions from FHF rat plasma. The top panel displays the Bio-Rad protein assay results in the fractions collected from DEAE anion-exchange chromatography. The elevated portion of the Bio-Rad protein assay response curve in the upper panel corresponds to the subpeak 7g in the lower panel of the chromatogram.

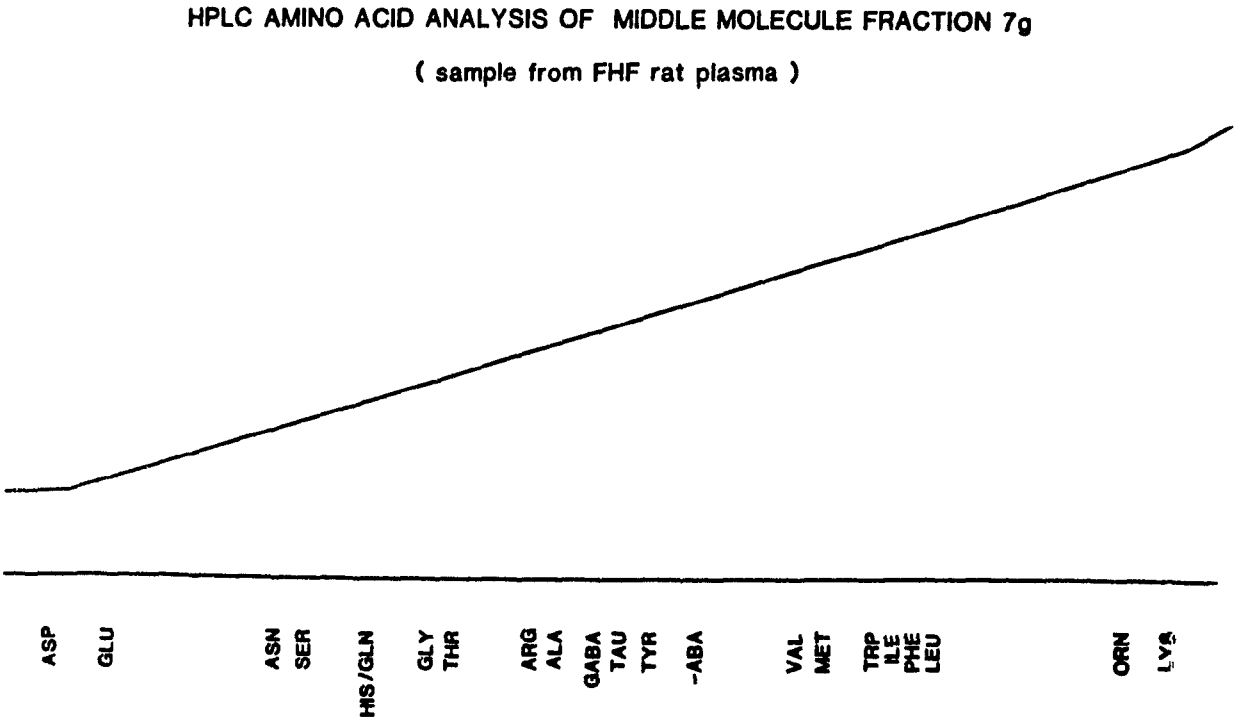


Fig. 3.4.4b. HPLC amino acid anaysis of subpeak 7g fraction separated from FHF rat plasma. This chromatogram shows that subpeak 7g, which contains high content of Bio-Rad protein dye-positive compounds, does not contain amino acids.

against the normal plasma samples which displayed no silver stain in the correspondent position in the electrophoresis gel.

The molecular weight of this peptidic band was estimated by the Polypeptidic Molecular Weight Standards (Pharmacia, Sweden). The relative migration values (Rf) of the standards and the unknown peptides were calculated according to the following equation:

$$Rf = \frac{\text{Distance peptide migrated from origin}}{\text{Distance from origin to reference point}}$$

The position of the tracking dye bromophenol blue was used as the reference point for calculation of Rf values. The standard curve was constructed on a semi-logarithmic graph paper with log MW as the ordinate against the relative mobility as the abscissa. The peptides of myoglobin digest composed a linear curve (Fig. 3.4.5b). The theoretical molecular weight of the middle molecules was read off the standard curve and estimated to be approximately 1,300 to 1,450 daltons (log MW: 3.11 to 3.16).

3.4.6. Beta-Endorphin Levels in Fulminant Hepatic Failure Rat Plasma and Brain

In this study, when all animals were sacrificed by decapitation without any anesthesia, there were no significant differences in plasma or brain beta-endorphin levels between the normal and the FHF groups. The plasma beta-endorphin level was 192.9 $\pm$ 75.4 pg/ml in normal rats, and 164.0 $\pm$ 86.4 pg/ml in FHF rats (Table 3.4.6a). The brain beta-endorphin level was

Lanes #1 #2 #3 #4 #5 #6 #7 #8 #9 #10

Fig. 3.4.5a. SDS-PAGE of plasma extract samples from normal and FHF rats. The lanes 1 and 10 contain polypeptide molecular weight calibration standards. The 2nd and 3rd lanes contain FHF rat (grade 2 coma) plasma extracts. The 4th, 5th and 6th lanes contain plasma extracts from FHF rats with grade 3 coma. The 7th, 8th and 9th lanes contain normal rat plasma extracts. The bands in the lanes 1 and 10 contain peptide standards with molecular wights of 17,201, 14,632, 8,235, 6,383, 2,556 and 1,695 respectively in the downward order.

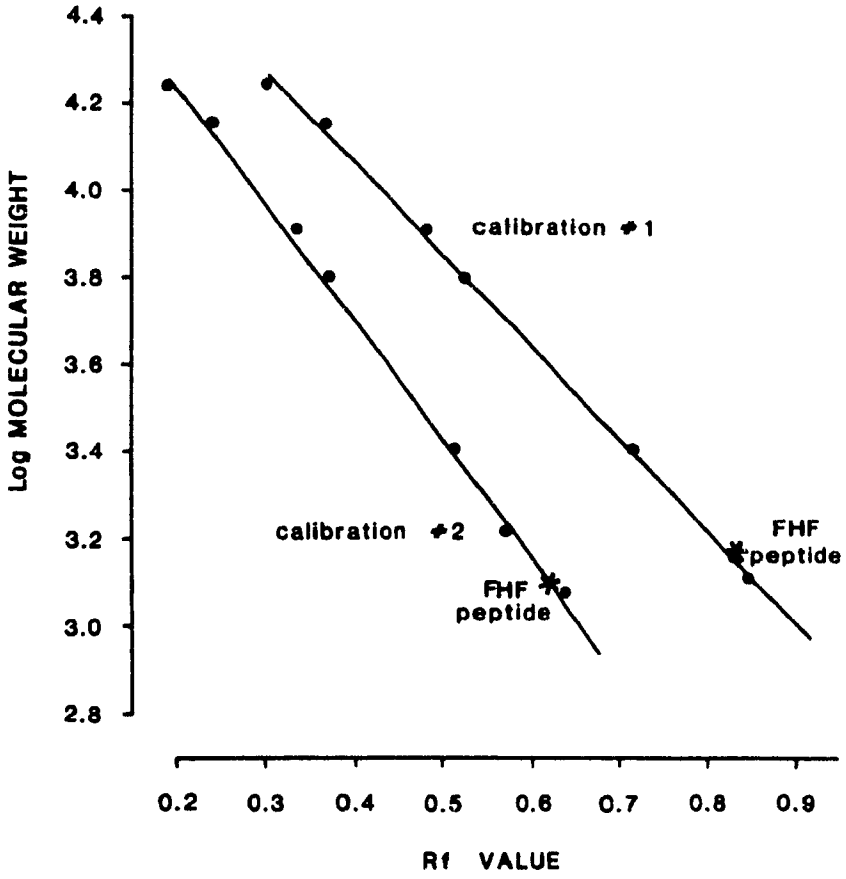


Fig. 3.4.5b. Molecular weight calibration of peptides in SDS-PAGE. The solid dots represent the peptide standards from partial cleavage of sperm whale myoglobin (product of Pharmacia Fine Chemicals). The * mark indicates the FHF peptide band in electrophoresis. The calculated log molecular weight of FHF peptide compounds is 3.11-3.16 which is approximately 1,300 to 1,450 daltons.

β-ENDOPOHIN LEVELS IN NORMAL & FHF RAT PLASMA

(ANALYSED BY RADIO-IMMUNO ASSAY)

(VALUES IN pg/ml PLASMA)

	NORMAL	FHF
	132.1	226.2
	286.2	109.7
	260.5	84.5
	154.8	143.4
	127.4	111.4
		309.0
MEAN	192.2	164.0
± S.D.	± 75.4	± 86.4

Student "t" test N.S.

Table 3.4.6a. Immuno-reactive beta-endorphin levels in normal and FHF (grade 3 coma) rat plasma samples. No significant difference is found between the two groups.

β-ENDORPHIN LEVELS
IN NORMAL & FHF RAT BRAIN

(ANALYSED BY RADIO-IMMUO ASSAY)

(Values in ng/gm tissue)

	NORMAL	FHF
	140.7	30.7
	16.7	11.4
	17.3	21.3
	14.4	21.4
	152.8	20.7
	115.5	42.7
MEAN	76.2	24.7
± S.D.	± 66.9	± 10.7
RANGE	14.4 – 152.8	11.4 – 42.7

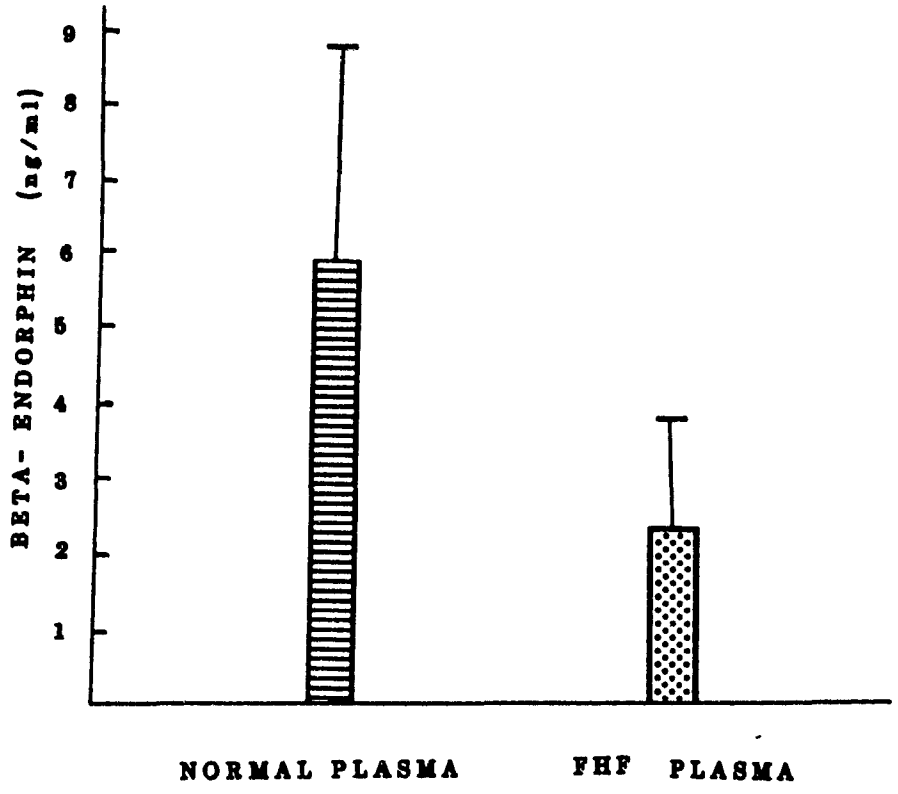
Student "t" test N.S.

Table 3.4.6b. Immuno-reactive beta-endorphin levels in normal and FHF (grade 3 coma) rat brain extract samples. No significant difference is found between the two groups.

Table 3.4.6c. IMMUNO-REACTIVE BETA-ENDORPHIN LEVELS IN PLASMA FROM ANESTHETIZED NORMAL AND FHF RATS

(Rats were anesthetized by Nembutal before sacrifice)
(Values in ng/ml, by RIA)

	Normal	FHF
Mean ± SD	5.88 ± 2.80	2.30 ± 1.48
n	6	12
Significance (t test)	$p < 0.05$	



76.2 ± 66.9 ng/g wet tissue in normal rats. This is comparable to the report of 108 ng/gm in normal rat brain by Rossier & Bloom (1982). A lower average level of 24.7 ± 10.7 ng/g was found in FHF rat brain (Table 3.4.6b). Although FHF rats seemed to have a lower average of brain beta-endorphin level, the whole range of variables (11.4 - 42.7 ng/g) was mostly within the normal range (14.4 - 152.8 ng/g). The difference was not statistically significant.

In the experiment with Nembutal anesthesia, beta-endorphin plasma levels in the FHF rats (grade III hepatic coma) were 2.30 ± 1.48 ng/ml which were significantly lower than the normal level of 5.88 ± 2.80 ng/ml (Table 3.4.6c). The FHF rat plasma level was only 40% that of normal level ($P<0.05$, by Student t-test). Normal rats responded to Nembutal anesthesia with an increase of beta-endorphin production, and the plasma beta-endorphin level was 30 times that of normal unanesthetized rats. The increase of beta-endorphin concentration in FHF rat plasma after anesthesia was only 14 times that of unanesthetized subjects.

This second experiment has led to the following two suggestions:

1) Beta-endorphin response to anesthesia is weakened in FHF rats;

2) Beta-endorphin is unlikely to be involved in the increased middle molecules in FHF rats.

3.4.7. Immuno-reactive Substance P in Fulminant Hepatic Failure Rat Plasma

Immuno-reactive substance P (i-SP) concentrations in FHF rat plasma was 12-fold higher than that in the normal controls (Table 3.4.7. and Fig. 3.4.7a). The normal i-SP level was only 70.5 $\pm$ 72.5 pg/ml while the FHF level was 878.8 $\pm$ 766.1 pg/ml in plasma. The difference between the two groups was highly significant ($P<0.005$).

Substance P is an undecapeptide (MW = 1,345). Its molecular size falls into the proposed molecular weight range of FHF middle molecules (500 - 2,000 daltons). When substance P was co-eluted with normal rat plasma in the G-15 gel permeation chromatography, the chromatogram showed that substance P raised the heights of peak 7 from 14 to 21 PHU, and peak 6 from 18 to 37 PHU. Both peak 7 and peak 6 were covered by middle molecule calibration standard Vit.B12 fraction. There were two other new peaks emerged at elution volumes of 10 - 11 ml and 15 - 17 ml, both being greater than the bed volume. They were marked on the chromatogram as peak 1 and peak 3 respectively (Fig. 3.4.7b). When the fractions were assayed with Bio-Rad Protein reagents, fractions from peak 7 and peak 6 displayed a highly increased positive reaction. The other fractions of peak 1 and peak 3 showed only negative reactions. Peak 1 and peak 3 fractions probably represent the other small non-peptidic solutes such as buffer salts contained in the substance P standard reconstitutes.

In the study of the chromatographic behavior of substance P in DEAE anion-exchange elution, substance P was found to be eluted at 2 - 4 ml elution volumes (Fig. 3.4.7c). This elution volume was different from that of subpeak 7g from FHF rat plasma and brain samples. Therefore, substance P was not included in the subpeak 7g, and was found to be significantly elevated in FHF rat plasma and brain. In other words, although subpeak 7g contained a fraction of the middle molecules, it may not necessarily represent all the abnormal middle molecules in FHF. Other subpeaks elevated in the anion-exchange chromatography may also comprise middle molecular substances.

Also shown in Fig. 3.4.7c. was the result of the in vitro adsorption of substance P by activated petroleum-based charcoal beads. After 2 hours of incubation with charcoal beads at room temperature, substance P in TRIS buffer solution was reduced by 72.5%. This reduction was calculated by comparing the substance P peaks on the chromatograms of DEAE anion-exchange chromatography.

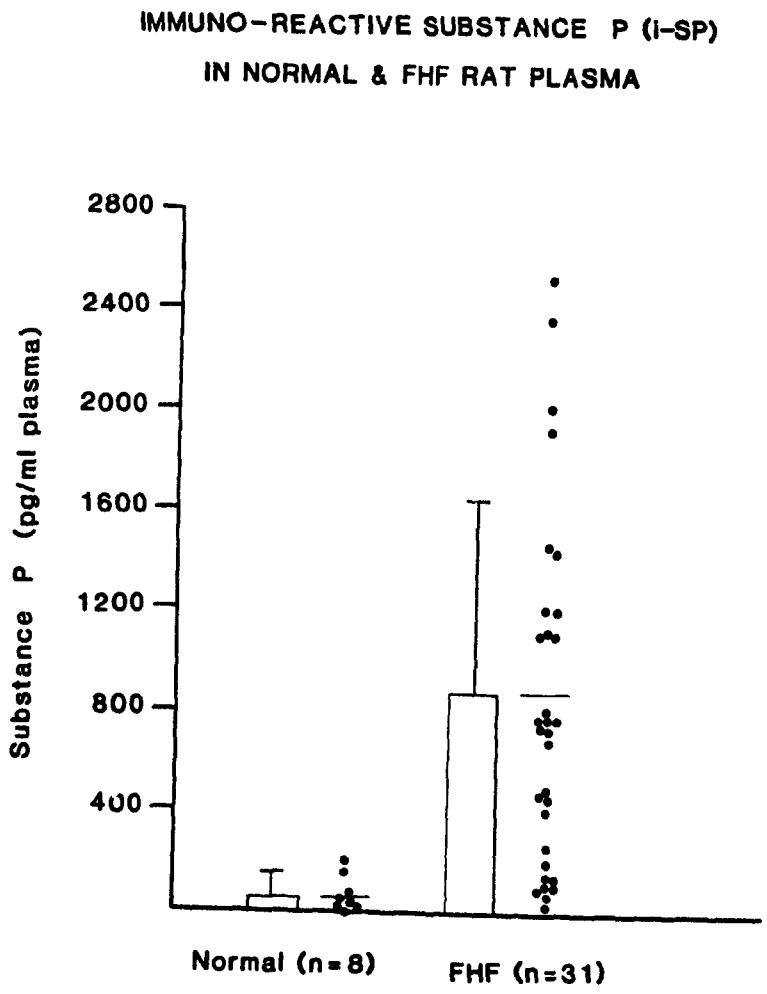


Fig. 3.4.7a. Immuno-reactive substance P (i-SP) concentrations (pg/ml) in normal and FHF rat plasma measured by radio-immuno assay.

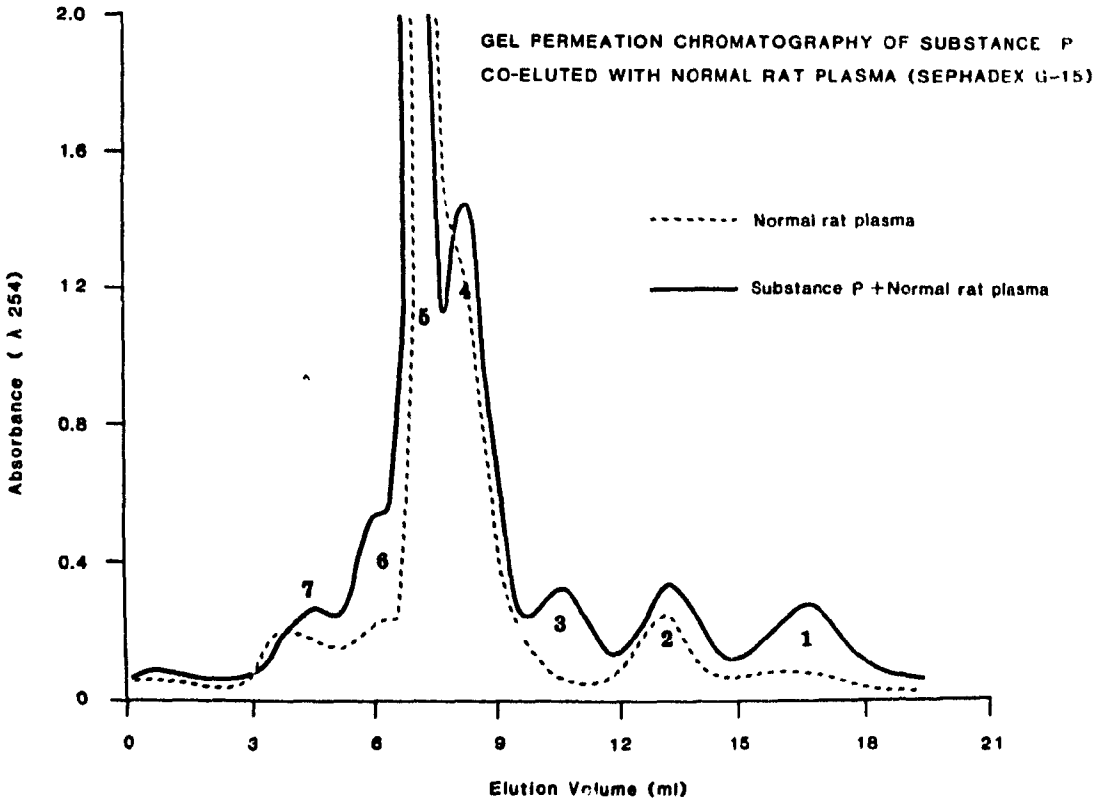


Fig.3.4.7b. Gel permeation chromatogram of substance P co-eluted with normal rat plasma. The peaks No. 7 and 6 in the plasma sample were found elevated after i-SP loading. The fractions from peaks 7 and 6 were found to contain substance P which was proved by the Bio-Rad protein dye reaction. Peaks 1 and 3 may contain the salts in the substance P standard reconstitutes.

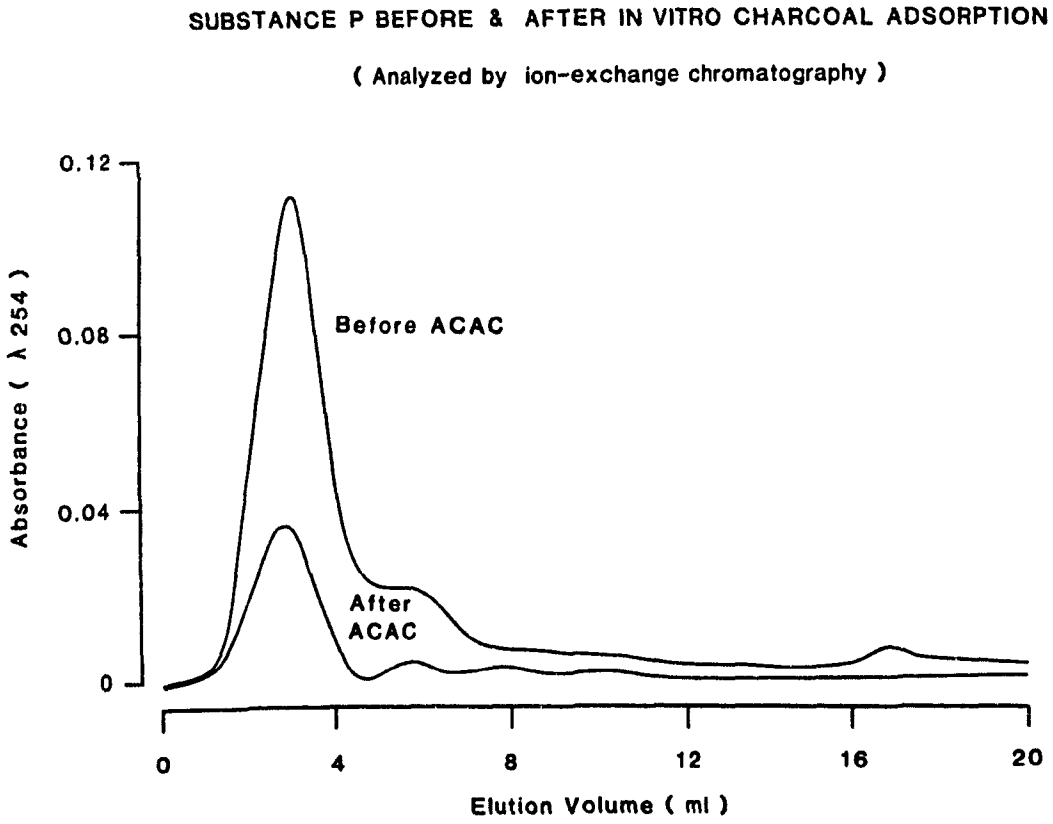


Fig. 3.4.7c. Substance P eluted in Sephadex DEAE A-25 anion-exchange chromatography. Substance P appears in the 3 to 4 ml of elution volume. Collodion coated activated charcoal markedly reduced substance P concentration in the in vitro adsorption experiment.

Table 3.4.7. IMMUNOREACTIVE-SUBSTANCE P CONCENTRATION IN NORMAL AND FHF RAT PLASMA

(pg/ml)

	Normal	FHF
Mean	70.5	878.8
S.D.	72.5	766.1
Range	0 - 200	10 - 2530
n	8	31
t-test	P < 0.005	

Immuno-reactive substance P was measured using the Substance P RIA Kit (Immuno Nuclear Corporation, Stillwater, Minnesota). The cross-reactivity for beta-endorphin and enkephalins are less than 0.008% and 0.002% respectively.

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CHAPTER 4

DISCUSSION

4.1. THE ABNORMAL PATTERNS OF AMINO ACIDS IN PLASMA, CSF AND TISSUES IN GALN-FHF RATS

4.1.1. Sources of the Elevated Amino Acids in FHF

In this GalN-FHF animal model with grade 3 hepatic coma, a severe state of catabolism and negative nitrogen balance was demonstrated. This is evidenced by the reduction in plasma protein levels, the accumulation of amino acids and middle molecule nitrogenous metabolites, and, to some extent, the loss of body weight.

As presented in Chapter 3 of this thesis, most amino acids (both essential and non-essential) were found to be significantly increased in the systemic and portal blood, CSF, Cerebral hemisphere, liver, kidney and the muscle. Essential amino acids are not synthesized in the mammal bodies. The fact that both essential and non-essential amino acids are found increased in this study is a strong evidence of protein breakdown being the cause of amino acid accumulation.

The major sources of the elevated amino acids in fulminant hepatic failure have never been clearly defined. Some hypotheses and suggestions have been made by a number of researchers.

(1) Amino acids released from the necrotic liver (Stadie &Van Slyke, 1920; Mason & Davidson 1924-5; Fischer, 1979).

(2) Increased protein catabolism in the peripheral organs (Folk et al, 1952; McMenamy et al, 1965).

(3) Decreased uptake and metabolism of the amino acids by

the liver (McMenamy et al, 1962; Miller, 1962).

(4) A decreased insulin/glucagon ratio, which stimulates a general catabolism further worsening the hyperaminoacidemia (Soeters & Fischer, 1976).

Frerichs (1860) first suggested over a century ago that a liver undergoing necrosis (autolysis) will release breakdown products into the circulation. He also suggested that the products of hepatic autolysis could produce a toxic effect in humans. This was confirmed later by some animal experiments. Mason and Davidson (1924) found that a portion of over 30 g of liver, when resected and left free in the peritoneal cavity of the dog, was associated with a rise in the blood levels of nitrogenous metabolites including amino acids. Stadie & Van Slyke (1920) confirmed Frerichs' discovery. They demonstrated that in a patient with acute yellow atrophy, liver protein underwent autolysis . The peptides and amino acids resulting from the hepatocellular autolysis enter into the systemic circulation. In his review on hepatic encephalopathy, Fischer (1979) concluded that "in acute fulminant hepatitis, severe hyperaminoacidemia of all amino acids is present and its source appears to be the necrotic liver".

The gastrointestinal tract has long been known to be one of the major source of nitrogenous substances, especially in the case of hepatic failure. Dietary protein and nitrogenous compounds are digested in the intestinal lumen by the proteinases, peptidases and bacteria. A great many nitrogenous substances, including peptides, amino acids, ammonia, amines,

etc., are thus produced. These substances are absorbed in the gut and enter the portal circulation. Among them, there are compounds which may interfere with normal brain functions and cause neuropsychiatric symptoms. Normally these substances are metabolized , detoxified, or excreted by the liver. But the impaired liver function in fulminant hepatic failure will cause the incomplete metabolism, and the accumulation of these nitrogenous substances.

Abnormal hepato-systemic circulation is also believed to contribute to the incomplete metabolism and detoxification of nitrogenous compounds in both fulminant and chronic hepatic failure (Sherlock, 1975). In patients with poor hepato-cellular function, such as FHF, the shunt is through the liver itself. The damaged cells are unable to metabolize the contents of the portal venous blood completely so that they pass unaltered into the hepatic veins and the vena cava. In patients with chronic liver diseases, such as cirrhosis and portal-hypertension, the portal blood bypasses the liver through large "collateral" vessels. The portal-hepatic venous anastomoses, developing around the nodules in a cirrhotic liver, also act as internal shunts. The condition here is analogous to the Eck fistula in experimental animals developing neuropsychiatric disturbances when given a meat diet.

In my own research, the skeletal muscle appears to be the major source of the abnormally accumulated amino acids in fulminant hepatic failure induced by GalN. As shown in Table 3.2.6., Figures 3.2.6. and 3.2.11., the skeletal muscle is

shown to have the most profound disturbance in amino acid levels. This is proven by the analysis of the several factors involved. First of all, the total mass of the skeletal muscle accounts for 45.5% of the body weight of the animal (Caster et al, 1956). It has already been well documented that the skeletal muscle protein undergoes catabolism in fulminant hepatic failure (Davidson, 1979). Secondly, the total amino acid concentration in the muscle in FHF was increased by 37% which was far higher than that of the other tissues studied. The 6% increase of total amino acids in the liver, 27% increase in the brain, and 21% increase in the kidney were all significantly lower than that in the skeletal muscle in FHF. Thirdly, the net increase in the total amino acid pool in the skeletal muscle (2334.2 micromoles) was 40 to 100 times as great as those of the liver (43.8 micromoles), the brain (19.3 micromoles) and the kidneys (53.5 micromoles) in FHF. The net increase in total amino acid pool in FHF plasma was 204.5 micromoles which was only 8.7% as much as that of the skeletal muscle.

Thus the skeletal muscle should be considered as the major contributor of the elevated amino acids in GalN-induced fulminant hepatic failure in rats.

In analyzing the data of individual amino acids, it was further found that the increases of Tyr, Phe, Trp, Met, Val, Ile, and Leu were the greatest in the skeletal muscle as compared with the other tissues (liver, brain and kidney). The derangements of all these amino acids have been implicated in

the biochemical mechanism of impairment of normal neuropsychological functions. Several other amino acids also showed their highest increase in the muscle as compared to the other tissues studied. These amino acids include Asp, Asn, Ser, Thr and Orn.

Protein balance in skeletal muscle is of fundamental importance in the control of muscle size and also in the overall energy homeostasis of the organism (Goldberg et al, 1980). Under certain pathological circumstances, the mobilization of amino acids stored in muscle protein helps provide the organism with essential precursors for hepatic or renal gluconeogenesis and also for protein synthesis in the organs and tissues of higher vital importance. The breakdown of proteins in skeletal muscle has been found to be responsible for the accumulation of free amino acids in many pathological conditions in which a negative nitrogen balance is featured (Wilmore, 1983). After release from skeletal muscle, the levels of the amino acids will only moderately increase in the plasma if the gluconeogenesis process takes place effectively. However, in case of massive hepatocellular injury, such as in FHF, the gluconeogenesis will be severely compromised. The amino acids released from skeletal muscle will then accumulate and distribute in the various body compartments.

In conclusion, in GalN induced fulminant hepatic failure in rats, the musculature constitutes by far the greatest source of amino acid accumulation. The catabolism of muscular protein and a disturbance in insulin and glucagon metabolism may be

responsible for the overproduction of amino acids in the muscle. Liver necrosis and autolysis is probably the second major source which also contributes to a surplus of free amino acids. Decreased hepatic uptake and metabolism of amino acids, as well as the protein breakdown in other tissues (brain, kidney, etc.) further accentuates such accumulation.

4.1.2. The Distribution of Amino Acids in Vaious Tissues in Normal and FHF Rats

In this study, the distribution patterns of amino acids in various body fluids and tissues of normal and FHF rats were demonstrated.

In the normal animals, the tissue in which total free amino acid concentration is the highest is the kidneys. The second highest concentration of total amino acids is found in the liver. The total concentrations of free amino acids in the skeletal muscle and the brain are the third and fourth highest respectively in the normal animals. Plasma normally has a very low level of total amino acids. As presented in Table 3.2.11a. and 3.2.12., the analysis of normal distribution ratios showed that the total amino acid concentrations in the brain, liver, muscle and kidneys were 12.3, 16.8, 13.1 and 32.5 times as high as that in the plasma. CSF, however, contains the lowest level of total amino acids amongst others.

It was further demonstrated in this study that not only the total amino acid concentrations, but also the profiles of 22 amino acids differed from one tissue to another. For

example, the brain was found rich in glutamate, aspartate, taurine and GABA. The aromatic amino acids in the muscle were lower than those in the other tissues. Concentrations of individual amino acids were generally higher in the kidney than in the other tissues.

The amino acid distribution among different body fluids and tissue pools is characteristic of the respective compartment or tissue. All tissues and organs are not alike in their amino acid contents as illustrated in Sections 3.2.1. through 3.2.13. in Chapter 3. Different levels of individual free amino acids in the corresponding tissues or body fluids are physiologically maintained constant. Such a constancy of the amino acid levels is characteristic of the specific metabolism and functions of any specific organ, tissue, or body fluid.

The maintenance of the constant patterns of amino acid distribution in normal animals is largely due to the control of the amino acid transport across the cell membrane. There are at least three processes involved in the metabolic passage of amino acids: uptake, exit and exchange (Blasberg & Lajtha, 1966). These passage processes are governed mainly by the carrier mediated transport mechanisms (Oldendorf, 1973; Oldendorf & Szabo, 1976; Newsholme & Leech, 1983). Prior to intracellular metabolism in various tissues, amino acids are transported from the interstitial space across the cell membrane. The transport of amino acids requires the presence of carrier systems in the cell membrane. The intracellular

concentrations of amino acids are usually greater than those in the blood stream and interstitial space. Therefore, the transport of amino acids into most, if not all, cells is an active process which is usually associated with the operation of a sodium ion pump and is energy-dependent. Some of the carriers, however, mediates the facilitated diffusion rather than active transport (Lajtha, 1973; Oldendorf, 1973; Oldendorf & Szabo, 1976).

A recent report has summarized the existence of eight different systems (Newsholme & Leech, 1983). These are system A (for alanine, glycine, proline, serine and methionine); system ASCP (for alanine, serine, cysteine and proline); system L (for leucine, isoleucine, valine, phenylalanine, methionine, tyrosine and tryptophan); system Ly (for lysine, arginine, ornithine and histidine); dicarboxylate system (for glutamic acid and aspartic acid); beta-system (for taurine and beta-alanine); N system (for glutamine, aspartic acid and histidine); and gamma-glutamyl-system (for amino acid transport at the renal tubules, erythrocytes and probably brain cells). These carriers display overlapping specificities for the amino acid substrates (Newsholme & Leech, 1983).

The carrier mediated transport systems at the blood brain barrier govern a facilitated diffusion process which is different from the active transport mechanisms in the other tissue cell membranes. Facilitated diffusion is characterized by downhill movement, high permeability, stereospecificity, saturation and countertransport (or, exchange diffusion).

Three carrier systems have been identified which mediate the transport of acidic, basic and neutral amino acids respectively across the blood brain barrier.

The kidney tubules are equipped with active transport mechanisms to accumulate amino acids (Heins, 1972). The reabsorption of most amino acids from the glomerular filtrate is accomplished by the gamma-glutamyl cycle on the brush border of the renal tubules. Six enzymes are involved in this pathway. The central enzyme is gamma-glutamyl transferase which catalyses the transfer of a glutamyl residue from the tripeptide glutathione (gamma-glutamylcysteinylglycine) to the incoming amino acid to form gamma-glutamyl-amino acid and cysteinylglycine. The gamma -glutamyl- amino acid is transported across the membrane and the amino acid is then released in the cytoplasm by another enzyme gamma-glutamylcylclotransferase. The kidney serves to retain the amino acids within the organism. The active reabsorption mechanism thus generates a constant pattern of high amino acid concentration in the kidney.

The liver is known to play a central role in amino acid metabolism. Amino acids absorbed from the enteric tract are removed by the liver. The body is normally protected by the liver against any excessive changes in the amounts of free amino acids entering the systemic circulation. It was found that the key catabolic enzymes for the essential amino acids are restricted to the liver (Munro, 1974).

Skeletal muscle plays a vital role in regulating the total free amino acid pool in the organism (Goldberg et al, 1980). The musculature contains a dynamic protein reserve which can be increased or reduced in response to the anabolic or catabolic stimuli. Amino acids can be taken up or released from the skeletal muscle under various physiological and pathological circumstances (Munro, 1974). Uptake of amino acids and synthesis of protein in the muscle is facilitated by insulin. Amino nitrogen released from muscle is rich in certain amino acids, e.g., alanine and glutamine (Munro, 1974).

The brain is unique in its amino acid contents. It is characterized by the presence of compounds that are found mainly in brain, such as GABA, and is rich in glutamate and related compounds including glutamine, aspartate and glutathione (Lajtha, 1973). The access of amino acids to the living brain is restricted by the transport mechanisms located on the blood brain barrier. The brain amino acid levels are also influenced by the transport mechanisms at the interface membranes between CSF and brain, and, CSF and blood.

Plasma normally has a relatively lower level of total amino acids than most other tissues. Plasma reflects what has happened to the homeostasis of amino acid metabolism, and carries out the functions of transporting amino acids and distributing them throughout various tissues and organs of the body, e.g., the brain, the kidneys, the skeletal muscles, etc..

In the CSF, the concentration of total amino acids was only 29% that of plasma concentration. The low concentrations

of free amino acids are maintained by the active transport mechanisms at the choroid plexus, ependyma and arachnoid membranes (Lorenzo, 1974; 1975). These transport processes remove amino acids from the CSF to blood. A "sink" phenomenon is created in CSF which favours the efflux of amino acids from brain parenchyma thus protecting the brain from insults by accumulated metabolites (Lorenzo, 1975).

Hence, the maintenance of the normal amino acid distribution pattern in the organism is mainly due to: (1) metabolism, and (2) transport mechnisms of the respective organs and tissues.

This constant pattern of amino acid distribution in various body fluids and tissue pools was shown to be maintained to a considerable extent even under circumstances where major disturbances were produced to the homeostasis of the animal as a whole (Roberts & Simonsen, 1962). Metabolic challenges such as starvation, dehydration and deficiencies of potassium or vitamin A produced only slight changes in the patterns of amino acid distributions in various tissue pools.

In fulminant hepatic failure, however, a new distribution pattern of amino acids among all tissues is established. The central role that the normal liver plays in regulating the amino acid metabolism is lost. FHF causes a drastic disturbance in the biochemical homeostasis of the organism. Each organ or tissue responds to such a disturbance depending upon its characteristic way of metabolism and unique physiological function. The responses are therefore all

variable. The discrepancies in their responses to the high catabolism in FHF are the reasons for the observation in this study that increases in amino acid concentrations are organ- or tissue-dependent.

As already seen in Tables 3.2.11a. and 3.2.11b., the magnitudes of increase in amino acids were all different among various tissues in FHF rats. The ratios of the tissues or body fluids to the plasma were also studied and presented in this thesis. In general, these ratios were markedly decreased for an overwhelming majority of total amino acids. This was due to the fact that the increase in plasma amino acids were much greater than that in any other tissues studied. In spite of a 580% (5.8 fold) net increase in plasma total amino acids, the muscle, brain,kidney and liver displayed relatively limited increases of 37%, 27%, 21% and 6% respectively in their total amino acid concentrations.

The concentrations of amino acids in portal plasma were found not significantly different from those in systemic plasma. This observation seems to suggest that the gastrointestinal tract is not the major source of elevated amino acids in this GalN model of fulminant hepatic failure.

The concentration ratio of total amino acids between CSF and plasma was as low as 29% of that in the normal rats. In FHF rats this ratio was decreased even lower to 11% in spite of the 5.7 fold increase in plasma concentration of total amino acids. The CSF concentration of the total amino acids (1.0 μmole/ml) was only 2% that found in the brain (43.5 μmole/g) of

normal rats. In the FHF rats, the total amino acid concentration in CSF (2.66 μmole/ml) was still merely 5% as much as the brain concentration (55.2 μmole/g).

It is obvious that the steep concentration gradient between the plasma and the various cellular tissues and organs in normal animals is markedly reduced in FHF subjects. Concentration of amino acids in plasma represents that of extracellular compartment, while the amino acid contents of the liver, brain, kidney and skeletal muscle are mainly contained in the intracellular compartment. The reduced tissue/plasma concentration ratios thus imply the reduction in concentration gradient between the extracellular and intracellular compartments in their amino acid contents.

The analysis of the correlation between the plasma and the tissues in the amino acid concentration changes in this study showed that the increases of most amino acids in CSF, brain and muscle, and about half of the amino acids in the liver and kidney were significantly correlated with the increases in plasma amino acids. The increases of Thr, Ala Tyr, Val, Met, Trp, Ile, Phe, Leu, Lys, His and Gln in CSF, brain and skeletal muscle, correlated significantly to the increases in the plasma. The same is true for Asn and Ser in the CSF and muscle, Asp and Gly in the brain and muscle, Arg in the CSF and Orn in the muscle. The correlation of amino acids between the plasma and the tissues is best illustrated by the distribution of tyrosine in the plasma, the liver and the brain (Section 3.2.10.).

The correlation of the liver and kidney to the plasma in amino acid levels was less predictable. This is probably owing to the very unique ways of these organs in metabolizing amino acids.

There were a few exceptional amino acids which do not show any correlation between the plasma levels and the levels in some tissues. Glutamic acid levels, for example, tended to decrease in CSF and brain in FHF rats even though its plasma concentration was markedly elevated ($P<0.05$). The decrease of glutamic acid was found to be associated with a concurrent increase in glutamine concentration in the CSF and brain. This could be due to the increased synthesis of glutamine as a consequence of the accumulation of ammonia in the central nervous system (James et al, 1979). Arginine was found universally decreased in plasma, brain, liver, muscle and kidney. The decrease of arginine level in the liver tissue is probably due to the jeopardized Krebs-Henseleit urea synthesis cycle of which arginine is an important component. Arginine deficiency has been known to be associated with hyperammonemia, while arginine administration therapy is found to be effective in correcting hyperammonemia (Walser, 1982). The reason for the arginine decrease in extrahepatic tissues was still obscure, but the disturbance in ammonia metabolism and urea synthesis could be involved.

Alpha-amino butyric acid (alpha-ABA) is a metabolite from methionine. The significant increase of alpha-ABA is probably

caused by the augmentation of methionine in FHF (Loda et al, 1983).

The specific free amino acid patterns in different compartments and tissues have been studied by Roberts and Simonsen (1962). They stated that in a given species at a particular stage of development each normal tissue, including blood cells, has a distribution of free amino acids which is characteristic for that tissue. This has been confirmed in the study of extracts of tissues from human, monkey, dog, cat, guinea-pig, rabbit, rat, mouse, opossum, chicken, alligator, snake, turtle, frog, salamander, and a wide variety of marine organisms in their laboratory.

In this study, I demonstrated the altered amino acid distribution in systemic blood plasma, portal plasma, CSF, cerebrum, liver, kidney and skeletal muscle in the galactosamine induced fulminant hepatic failure in rats. My study of amino acid distribution in various tissues showed that tissue specificity existed in normal rats and, to a considerable extent, the FHF rats. It was also demonstrated that the significant increases of most amino acids in CSF, brain, liver, skeletal muscle and kidneys in FHF rats, were statistically correlated with, but to a much lesser extent than, the amino acid increases in the plasma. The ratios of amino acids in various tissues (skeletal muscle, liver, brain, kidney and CSF) to those in the plasma were markedly decreased in FHF as compared to those in the normal rats. In spite of the changes in these tissue/plasma ratios, the tissue- or

organ-specific patterns were still maintained to a considerably large extent.

4.1.3. Changes of The Aromatic Amino Acids and The Molar Ratio of BCAA/AAA

In this study, the increases in the aromatic amino acids were much more profound than most of the other amino acids. Tyrosine was almost always the most markedly increased and its increase was found in all tissue and body fluids studied. Phenylalanine was increased significantly in plasma (portal and systemic), CSF, brain and muscle but not in the liver and kidneys. Branched chain amino acids, valine, leucine and isoleucine were all found significantly increased in plasma (portal and systemic), CSF, muscle and brain, but not in the liver and kidneys. The increases of BCAAs were comparable to that of phenylalanine but were much less marked than that of tyrosine. Therefore, primarily due to the marked increase in tyrosine, the BCAA/AAA (Val+Leu+Ile/Tyr+Phe) molar ratios were significantly reduced in all tissues except the kidneys.

My finding of BCAA increases in FHF is quite different from Fischer's findings in portal systemic encephalopathy in which BCAAs are unchanged or even slightly decreased (Fischer, 1979). This is probably due to the much more profound and acute protein catabolism in FHF than in cirrhosis and portal systemic encephalopathy studied by Fischer's group.

The reason for the heterogeneous alterations of AAA and BCAA has not been very clear. Several possibilities were

suggested to influence the plasma aromatic amino acid concentrations (Fischer, 1979). First the exogenous intake contributes at a considerable extent to the elevation of plasma levels of aromatic amino acids. It has also been found that the nitrogen balance correlates with the plasma and brain levels of aromatic amino acids. A negative nitrogen balance in animal causes plasma AAAs to rise up.

The uptake of BCAAs by the extrahepatic organs for glyconeogenesis may partially account for this observation (McMenamy et al, 1962; Herlong & Miehl, 1982). The experiments with isolated perfused dog liver showed that the liver had little ability to remove the BCAAs from the perfusate. The uptake of other amino acids by the same perfused liver was much higher than that of BCAAs. The reason for such a difference between the liver and the non-hepatic tissues in the relative uptake of amino acids was believed to be due to the slower rate of oxidation of the BCAAs by the liver compared with the extra-hepatic tissues (Miller, 1962). Branched chain amino acids are metabolized in the extrahepatic tissues such as skeletal muscle and fat tissues. But aromatic amino cids which are ordinarily metabolized in the liver would escape degradation in case of liver dysfunction (Herlong & Miehl, 1982). In hepatectomized dogs, the plasma concentration of leucine, isoleucine and valine were found to be decreased, but all other amino acids were increased (McMenamy et al, 1965). In this GalN-induced fulminant hepatic failure, although BCAAs are increased, the extrahepatic uptake and consumption of BCAAs cause the BCAAs

to increase at a less marked magnitude than that of AAAs. The ultimate effect on reducing BCAA/AAA molar ratio is the same in these conditions.

4.1.4. GABA in Blood and Brain of FHF Rats

My study now gives the first report on the significant elevation of GABA concentrations in the liver, kidney and some brain regions (cerebrum and brain stem) in fulminant hepatic failure animals induced by GalN. The significant increase in GABA concentration may be one of the major contributing factors in causing the neurological deterioration in fulminant hepatic failure.

GABA has been shown to inhibit single neurons (Krnjevic 1963). Of particular clinical interest is the suggestion that most tranquillizing drugs, including alcohol, barbiturates, and benzodiazepines, may act by increasing the effectiveness of GABA at postsynaptic receptors (Cowen & Nutt, 1982). It has also been shown that cerebral administration of GABA can induce coma. When conscious rabbits received instillation of less than 1 μmole of GABA into the hippocampal region, they became quiet and lost spontaneous locomotor activity. Within 10 seconds, the animals mostly lied on the cage floor (Smialowski, 1978). These behavioral anomalies are associated with spreading delta waves in electroencephalographic recordings. The EEG waves are similar to those reported in patients or animal models with hepatic coma (Blitzer et al, 1978; Trewby et al, 1978).

When Schafer and Jones (1982) proposed the concept of GABA neuroinhibition in hepatic coma, they demonstrated the increased GABA levels in portal blood and systemic blood. In their GalN rabbit model of fulminant hepatic failure, they found that gut-derived GABA penetrated blood brain barrier and induced a higher brain sensitivity to GABA-ergic neural inhibition (Schafer & Jones, 1982).

According to the founders of the "GABA neuroinhibition theory" (Schafer & Jones, 1982), a feasible pathophysiologic basis for hepatic encephalopathy is the increased inhibitory neurotransmission, and the decreased excitatory neurotransmission. These two types of neurotransmissions were mediated by the respective amino acid neurotransmitters. GABA neuroinhibition may result from altered GABA receptors and GABA metabolism within the brain. The possibility that GABA neuroinhibition is mediated by an unidentified GABA agonist in liver failure is also not excluded (Jones et al, 1984).

The heterogeneous distribution of GABA in rat brain regions has also been demonstrated in this study. This is supported by earlier studies (Enna, 1978). The neurophysiological, biochemical and histochemical analyses have indicated a heterogeneous distribution of the GABA-ergic system in mammalian brains (rat, monkey and human). It has also been suggested that the receptor affinity for GABA may vary significantly among different brain regions. Subtle alterations in the distribution and affinity of GABA receptor

can lead to dysfunction of the brain region involved (Enna, 1978).

GABA concentrations in plasma, CSF and skeletal muscle (ranging from fMol to pMol) were too low to be detected using the present HPLC techniques. Most studies of GABA in these body fluids and tissues were carried out using radio receptor assays (Löscher, 1982)

The abnormal increase in GABA concentration in the liver and kidney tissues in fulminant hepatic failure has never been reported. The pathological significance of GABA increase in these two organs requires further investigation.

4.1.5. Free and Total Tryptophan in Plasma and Brain of FHF Rats

This study demonstrated the significant tryptophan increase in both protein-bound and unbound (free) forms in plasma of GalN-FHF rats. In association with the plasma level, the tryptophan levels in CSF and brain were all significantly raised. Tryptophan content in the brains of normal and FHF rats was proved to be almost exclusively in the free (unbound) form.

Tryptophan has been reported to induce hepatic encephalopathy in dogs with Eck fistula in the absence of hyperammonemia (Ogihara et al, 1966). Sherlock (1975) reported the neurological deterioration in patients with hepatic disease after oral ingestion of tryptophan. The accumulation of tryptophan may be associated with altered metabolism of

serotonin, tryptamine and indolic metabolites in the central nervous system (Lal et al, 1974; Marsden & Curzon, 1978; Sourkes, 1978; Young & Lal, 1980).

Tryptophan is unique among amino acids since it is transported mainly in the albumin-bound form in the blood. Under normal circumstances, about 90% of the tryptophan in blood plasma is bound to albumin, and only about 10% is in the unbound form which is freely exchangeable with the free tryptophan in the tissues (Curzon & Knott, 1974). Any factor that disturbs the equilibrium of tryptophan binding or varies the amount of tryptophan that is freely diffusible, would affect its uptake by the brain and other tissues. A large number of substances, e.g., hormones, drugs and fatty acids, also bind to albumin in the blood. An increase in the binding of one compound may cause the displacement of another. Other albumin-bound substances may competitively displace tryptophan from its albumin-binding sites. The concentration of unbound tryptophan is increased by the administration of these competitive albumin-bound substances even though the total tryptophan level (free and albumin-bound) may still remain unchanged. It has been shown that following either salicylate ingestion, or elevation of serum non-esterified fatty acids, the brain concentrations of tryptophan and serotonin are significantly increased (Curzon & Knott, 1974; 1977; Gessa & Tagliamonte, 1974).

The significant increases of free tryptophan in the brain and CSF correlated with the augmented plasma concentration of

free tryptophan (Young et al, 1976). The rise in plasma free tryptophan may be due to: (1) increased release of tryptophan from muscle degradation; (2) reduced hepatic uptake and metabolism; and (3) competitive displacement by other albumin-bound substances, such as aspirin and non-esterified short chain fatty acids (Curzon & Knott, 1974).

Free tryptophan in plasma is capable of equilibrating with tryptophan in other body compartments (Young et al, 1976). The tryptophan transport system across the blood-brain-barrier was found to be comprised of two kinetically distinct components (Mans et al, 1979). One component is low in capacity, saturable and obeys Michaelis-Menten kinetics (normal: Vmax = 19.5 nmol/min/g, Km = 113μm; FHF: Vmax = 33.8 nmol/min/g; Km = 108 μm). This low component transports several neutral amino acids and the transport of tryptophan is therefore reduced by competitive inhibition. The second component is a high capacity system which transports tryptophan in direct proportion to concentration gradient. It was shown by Mans et al (1979) that in FHF transport via both components was increased substantially, approximately doubling the normal rate of tryptophan penetration of the blood-brain barrier. Experiments have demonstrated that the increased competing amino acids in plasma would only cause a reduction in tryptophan transport by the low capacity system. The contribution by the high capacity component became even more significant than in normal rats, accounting for 75% of all tryptophan passage across the blood-brain barrier. The net

tryptophan entry to the brain is much augmented and consequently, the brain tryptophan concentration rises significantly.

The increased tryptophan level in the brain can result in an increased synthesis of serotonin. Serotonin is an inhibitory neurotransmitter which may participate in the precipitation of hepatic coma. Walshe (1953) also noted that a number of indole derivatives from breakdown of tryptophan were more toxic to brain in vitro than ammonia. They possessed depressive, excitatory, or hallucinogenic effects. Some indolyl compounds disturb the metabolism in rat brain slices which responded to potassium chloride (used to stimulate cell respiration) only about 1/3 as well as brains from normal animals.

Although many experimental data showed that administration of tryptophan in dogs and humans could cause neuropsychiatric symptoms (Ogihara et al, 1966; Sherlock, 1975), its direct relationship with hepatic coma was not clear. Recently, the elevated CNS tryptophan metabolism was found to be associated with hepatic coma in cirrhosis (Young & Lal, 1980). A strong correlation was illustrated between the grade of hepatic coma and CSF indoleacetic acid which is the metabolite of tryptophan. However, when the concentrations of tryptophan in CSF were compared between the cirrhotic patients in coma and those not in coma, no significant difference was found (Sourkes, 1978).

My study has given the positive evidence of marked increase in free and protein bound tryptophans in plasma, and the increase in free tryptophan in the brain in fulminant hepatic failure. However, the exact role of tryptophan in the pathogenesis of hepatic coma still awaits further clarification.

4.1.6. Tyrosine in Plasma, Liver and Brain of FHF Rats

The measurement of tyrosine levels in normal rats and FHF rats with various grades of coma demonstrated the progressive accumulation of aromatic amino acids in association with the progression of hepatic failure and coma. The simultaneous measurements of tyrosine levels in serum, liver and brain provided positive evidence of correlation between the aromatic amino acid in circulation and those in the organ tissues.

The results of tyrosine changes in blood and brain compartments in rats with various grades of coma were in close agreement with the results obtained in the HPLC analysis of amino acids in normal and FHF rats. In the previous experiments using HPLC analysis (Sections 3.2.1. through 3.2.7.), only normal and grade 3 coma rats were studied for amino acid changes in the plasma and tissue pool. While in this study, normal rats and rats with grade 1 to grade 4 coma were all included. Thus, the correlations of tyrosine concentrations in plasma, brain and liver tissue pools to the severity of hepatic coma were best illustrated.

In the previous results (Sections 3.2.1. through 3.2.7.), I concluded that in grade 3 hepatic coma rats, the increases in amino acids in all tissues studied were correlated to, but at a much lesser extent than, those in the plasma. This is best evidenced by the distribution and the relative concentrations of tyrosine in the plasma, the liver and the brain. In this experiment, the manually performed fluorometric assay of tyrosine in plasma, brain and liver tissues also revealed a similar relationship between the plasma and the tissue tyrosine concentration. The brain:plasma ratio of tyrosine concentration in normal rats was 1.3:1. During grades 2 and 3 coma, the ratio was reduced to 0.75:1. Thus, in the normal rats the brain tyrosine was much higher than the plasma tyrosine level. However, in FHF rats with grade 3 and 4 coma, brain tyrosine level was significantly lower than the plasma level. These results suggested that the amino acid carrier transport systems were still maintained effective. If this were not the case, the plasma tyrosine concentration would equilibrate into the cerebral milieu. There has been some recent experimental evidence pointing to the increased transport of the aromatic amino acid transport across the blood brain barrier in portal systemic encephalopathy (James & Fischer, 1981; Hawkins et al, 1983). In fulminant hepatic failure, however, the situation is probably different because of the increased permeability of the blood brain barrier (Livingstone et al, 1977; Horowitz et al, 1983; Zaki et al, 1984). Thus the increased brain amino acids could be partially

attributed to the increased permeation across the blood brain barrier. However, it was also found in this study that the concentrations of cerebral aromatic amino acids are lower than those in the plasma even in the late stages of hepatic coma (grades 3 and 4). This finding is likely to indicate that the amino acid transport mechanisms in the brain are probably maintained active to certain extent in fulminant hepatic failure. The net effects of these events were that the aromatic (and others) amino acids were increased in the brain but to a limited magnitude.

The detailed investigation of tyrosine changes in this study can be considered as representative of the other aromatic amino acids in their concentration and distribution changes in FHF. Thus, at least two suggestions can be made from this study of aromatic amino acids:

(1) Aromatic amino acids in the brain are increased in correlation with the increase in the plasma, but the magnitude of increase in the brain is much smaller than that in the plasma;

(2) Brain aromatic amino acids in FHF rats with grade 3 and 4 coma were significantly lower than those in the plasma. This is probably due to the amino acid transport mechanisms which control the bidirectional passage of the amino acids including aromatic amino acids (Blasberg & Lajtha, 1966; Newsholme & Leech, 1983).

4.2. EFFECTS OF REDUCING PLASMA AMINO ACID LEVELS BY HEMOPERFUSION ON THE AMINO ACID LEVELS IN CSF AND BRAIN

4.2.1. Preferential Removal of Aromatic Amino Acids by Activated Charcoal Hemoperfusion

Charcoal's enormous surface area to mass ratio makes it extremely potent for adsorption of a great variety of substances. It has been discussed in the earlier sections of this thesis (Section 1.5.2.3.) that as a general rule, non-polar or hydrophobic solutes are better adsorbed from the aqueous solution than polar hydrophobic solutes (Denti & walker, 1980). The magnitude of amino acid removal in this study, by and large, corresponds to the hydrophobicity of the individual amino acid.

The difference in the affinity of charcoal surface to various amino acids was first noted in the early 1940's, when charcoal began to be used as a means of chromatographic solid support in separating amino acids (Cheldelin & Williams, 1942). When charcoal columns were loaded with amino acid mixture solution, aliphatic amino acids could be eluted readily with 5% acetic acid. The three aromatic amino acids, tyrosine, phenylalanine and tryptophan remained firmly on the column after acetate elution. They could only be subsequently eluted by the use of 5% phenol in 20% acetic acid. Tiselius further advanced the charcoal adsorption chromatographic techniques in separating amino acids and summarized the retentivity of more than 10 amino acids and some oligopeptides on activated charcoal column (Tiselius, 1944). Thus, in his adsorption

liquid column chromatographic separation, the increasing order of the retention volumes of the amino acids were alanine, OH-proline, proline, valine, leucine, isoleucine, methionine, histidine, arginine, phenylalanine and tryptophan. The larger the retention volume, the greater the degree of adsorption of the compound on the charcoal column. Here, alanine and OH-proline being polar amino acids were readily washed off during the early elution, whereas phenylalanine and tryptophan being aromatic amino acids bearing hydrophobic side groups were retained much more firmly by the charcoal surface.

Recently, Zaslavsky and co-workers studied the relative hydrophobicities of the amino acid side-chains and established a new hydrophobicity scale for apolar and ionogenic side-chains of the amino acids (Zaslavsky et al, 1981). The amino acids they studied displayed a relative hydrophobicity scale which was very similar to the order of retention volumes in charcoal adsorption chromatography presented in this thesis and reported earlier (Shi & Chang, 1983). With the increasing hydrophobicities, the amino acids were ranged in the order of: glutamic acid, aspartic acid, asparagine, serine, glycine, glutamine, proline, threonine, alanine, valine, methionine, arginine, isoleucine, leucine, lysine, phenylalanine and tryptophan. There was a slight difference in the elution orders between Tiselius' study and Zaslavsky's study. This was due to the fact that Zaslavsky used dinitrophenylated amino acid derivatives rather than amino acids per se. In addition to the hydrophobicity of the amino acids, the ionic and

nonionic polar side groups of the amino acids may also influence the elution order by varying the interaction between the amino acids and the charcoal surface. Even taking all these factors into account, it still remains valid that the charcoal surface has a greater affinity for, and hence removes much more, amino acids with higher hydrophobicity than those with low hydrophobicity.

In this study, coated-charcoal hemoperfusion has substantially reduced blood levels of the aromatic amino acids tyrosine, tryptophan, phenylalanine and some other amino acids such as methionine and histidine which possess side groups of relatively higher hydrophobicities. The removal of other neutral amino acids such as branched chain amino acids is significant but smaller. The polar amino acids including acidic and basic amino acids were less significantly affected by charcoal hemoperfusion. Both in vitro and in vivo experiments demonstrated the preferential removal of aromatic amino acids by charcoal adsorption. Such removal in fulminant hepatic failure favours a correction of the deranged molar ratio of BCAA/AAA.

4.2.2. Changes of Amino Acids in CSF and Brain Following the Reduction of Plasma Amino Acids by Hemoperfusion

In comparison to the control experiments, coated charcoal hemoperfusion resulted in significant removal of plasma neutral amino acids, including aromatic and branched chain amino acids. The effects of the lowering of plasma neutral amino acids on

the levels of these amino acids in CSF and brain were presented in Sections 3.3.1. through 3.3.5. and are summarized as follows:

Aromatic amino acids (AAA) in CSF were significantly reduced in rats receiving 2 consecutive 1-hr hemoperfusions. On the other hand, the removal of AAAs in 1-hr hemoperfusion was not sufficient enough to significantly lower the AAA levels in CSF.

In the brain cortex, neither 1-hr, nor 2 consecutive 1-hr CAC hemoperfusions resulted in any significant reduction of any of the amino acids studied. Thus the significant reduction of AAAs in the plasma compartment by CAC hemoperfusion exerted no obvious influence on the brain compartment.

Tyrosinase artificial cell hemoperfusion was chosen as an effective means to selectively remove a major aromatic amino acid, tyrosine. The clearance of plasma tyrosine was very high and the total removal of plasma tyrosine was so great that the plasma tyrosine level dropped to only 40% of the initial level. Even then, the brain tyrosine concentration was not affected to any significant extent.

The reasons for the above observations are complex. The recent studies have concluded that the efficiency of solute removal from the body is governed not only by rates at which plasma is cleared of the solute, but also by solute transfer rates and distribution ratio within body compartments (Farrell & Skalsky, 1980; Gibson, 1980). In order to evaluate the effects of hemoperfusion on amino acids in blood, CSf and

brain, the following hemoperfusion parameters are analyzed:

(1) Plasma clearance;

(2) Volume of distribution;

(3) Compartmental transfer in the brain.

4.2.3. Plasma Clearance of Amino Acids in Hemoperfusion

The concept of plasma clearance in hemoperfusion is identical to the concept of creatinine clearance in hemodialysis and is simply the volume of plasma from which a specified quantity of a substance is removed per unit time. Thus, the greater the plasma clearance, the more efficient the hemoperfusion in removing the unwanted substances.

The amino acid clearance in activated charcoal and tyrosinase artificial cell hemoperfusions are quantitatively analyzed.

It was demonstrated that the CAC (collodion coated activated charcoal) has a higher affinity for AAA than for any other amino acids. The extraction rates were in the range of 60% to 90% in a single passage of the blood through the hemoperfusion shunt. The in vitro and in vivo removals of AAA Tyr, Phe and Trp by charcoal hemoperfusion were the greatest of all amino acids. The total amount of aromatic amino acids removed was calculated based on the factors of clearance and flow rate in CAC hemoperfusion. The calculation and the results were tabulated (Table 4.2.3.). The calculated total clearances are expressed as the number of pre-hemoperfusion plasma pools that were cleared. It can be clearly seen that 2 hrs CAC

Table 4.2.3.

TOTAL CLEARANCE OF PLASMA AROMATIC AMINO ACIDS

TOTAL BLOOD VOLUME	18 ml
TOTAL PLASMA VOLUME	10.2 ml
FLOW RATE	0.34 ml/min plasma (0.6 ml/min blood)
EXTRACTION RATE	70% (on average)
CLEARANCE RATE	0.34 ml/min X 70% = 0.24 ml
TOTAL CLEARANCE	0.24 ml/min X 120 min = 28.8 ml
NUMBER OF PLASMA POOLS CLEARED	28.8 ml / 10.2 ml = 2.8 (pools)

Notes: The method of determining the plasma volume (PV) is adopted from the Geigy Scientific Tables (1970):

$$BV = 100 \times (PV/100-Ht)$$

Where BV is the blood volume, PV is the plasma volume, and Ht is the hematocrit:

$$Ht = HtV \times 0.97 \times 0.91 = HtV \times 0.88$$

Where HtV is the venous hematocrit. The factor 0.97 allows for the trapped plasma remaining in the erythrocyte column after centrifuging (micro-hematocrit technique). The factor of 0.91 allows for the lower erythrocyte content in the blood as a whole than in venous blood. In FHF rats, the measured average HtV was 50%. The total blood volume was calculated as:

$$\text{Blood Volume} = \text{Body weight} \times 6\% = 300\ g \times 6\% = 18\ ml$$

hemoperfusion removed the equivalent of 2.8 plasma pools for each of the aromatic amino acids.

The total clearance of tyrosine during the 2-hrs tyrosinase artificial cell hemoperfusion can be approximated as the product of clearance (ml/min) multiplied by the total hemoperfusion time (min). The clearance at the middle point of the 2-hrs hemoperfusion procedure was 0.3 ml plasma/min and the total hemoperfusion time was 120 minutes. Thus the total clearance should be:

Total Clearance = 0.3ml plasma/min X 120 min = 36ml plasma

As illustrated in Table 4.3.2a, the total plasma volume in a 300 g male rat is approximately 10.2 ml. When the total clearance is calculated as the number of pre-hemoperfusion plasma pools that were cleared of a given amino acid, then:

36 ml / 10.2 ml = 3.53 (pools)

Thus 2 hours hemoperfusion removed an equivalent of 3.43 plasma pools of tyrosine.

It is obvious that the plasma clearances are very high for the removal of aromatic amino acids in charcoal hemoperfusion and tyrosine in tyrosinase artificial cell hemoperfusion. The plasma clearances analyzed here seem to favor a removal of aromatic amino acids from the total body pool of amino acids.

4.2.4. The Volume of Distribution of the Aromatic Amino Acids

Volume of distribution (Vd) is originally a pharmacokinetic concept that views the body as a homogeneous reservoir of water. The apparent Vd is the amount of water

that the total amount of a given solute must have been dissolved in to give the observed plasma concentration (Gibson, 1980). Vd is approximated by the equation:

$$Vd = \frac{\text{Total amount of solute}}{\text{Plasma concentration}}$$

The apparent Vd (liter) may be smaller or greater than body weight (kg). An apparent Vd greater than body weight indicates there are tissues other than plasma that have a higher concentration of that solute. Volume of distribution is an important factor in determining the efficiency of the hemoperfusion system in removing a given solute from the body as a whole. It is generally agreed that solutes with large volumes of distribution cannot be readily removed by hemoperfusion because major portionof the drug is inaccessible to the plasma and the hemoperfusion system (Petit & Biggs, 1977; Goulding, 1978). The efficiency of hemoperfusion in removing solutes with large Vd is low because the majority of the solute is inaccessible to the plasma and the hemoperfusion cartridge.

Even though the high plasma clearance favors a removal of AAA from the tissues, the analysis of the volume of distribution of the AAA (as well as other amino acids) is found to be a negative factor against such a removal. As shown in sections 3.2.11., the volume of distribution of amino acids is extremely great. In the normal rats, the total free amino acid pool in the muscle mass is 174 times as great as that in the plasma. In FHF rats, the total muscle free amino acid pool is

still 36 times as great as the plasma pool. Taking into account the amino acid pools in other tissues including the liver, kidneys, gastrointestinal tract and other organs, the total volume of distribution of free amino acids in the body as a whole is probably more than 50 times as much as that in the plasma. Such a large volume of distribution forms an apparent obstacle to the removal of amino acids form the tissues even though amino acids are rapidly and constantly removed from the plasma by hemoperfusion.

4.2.5. Compartmental Transfer of Amino Acids in Hemoperfusion

Hem operfusion, just like hem odialysis, has direct access only to the blood pool but only indirect access to the tissue pools (Schindhelm & Far rell,1978). The tissue pools may be an order of magnitude larger in volume than the blood pool. The blood and tissue pools will, however, be in some kind of equilibrium with each other such that reducing the con centration in the blood pool should cause a reduction in the tissue pool. Therefore, the solute removal from the body is governed not only by plasma removal rates, but also by int erc ompartmental transfer of the solutes. Slow tis sue/plasma mass transfer rates have been shown to limit in vivo performance of hemodialysis (Schindhelm & Farrell, 1978).

Single or multicompartmental models have been proposed for the removal of different solutes in hemoperfusion and hemodialysis (Schindhelm & Rarrell, 1978; Gibson, 1980; Farrell & Skalsky, 1980). A 1-compartment (homogeneous body water) model has been proposed for urea removal, and a 2-compartment (intracellular and extracellular) model for creatinine removal in hemodialysis.

Amino acids have been considered to distribute into 2 compartments, the extracellular and intracellular fluids (Coulson et al, 1975). This model may be appropriate for most extracerebral tissues, since the passage of small molecule solutes out of the blood capillaries of these tissues is extremely rapid (Davson, 1967). The delay of tissues in

reaching equilibrium with plasma is probably entirely due to the time required for passage into the cells. It is the membrane of the cells that exhibits a barrier to diffusion. In skeletal muscle and many other tissues (may be treated as a 2-compartment model) the composition in the interstitial fluid is very close at all times to that in the plasma, so that the rate-limiting factor in the kinetics of passage from blood to tissue-water is the passage from extra- to intra-cellular compartment (Rapoport, 1976; Granger & Perry, 1983).

In the brain, however, the system is more complex because of the intervention of the blood brain barrier which is composed of endothelial cells joined together by tight junctions. Thus the exchange of the solutes between plasma and the interstitial fluid in the brain is restricted by the barrier. The solute uptake by the brain is therefore the result of two restraints on transfer, namely from blood to extracellular space and from extra- to intracellular space (Davson, 1967; David, 1975; Rapoport, 1976).

Owing to the unique capillary structure, the blood brain barrier, the brain has been studied using a multicompartmental model (Levin & Patlak, 1972). Plasma in the cerebral vasculature is one compartment. The brain is divided into an extracellular compartment that is approximately 20% of brain wet weight and an intracellular compartment corresponding to approximately 80% of wet weight (Rapoport, 1976). Thus any variation in the plasma concentration of a given solute will affect the extracellular concentration first, and thereafter,

the intracellular compartment.

In the interpretation of amino acid transfer between the brain compartments, the amino acid transport systems need to be emphasized. Amino acids are transported at the blood brain barrier, choroid plexus and the neuronal and glial cells membranes (Oldendorf, 1973; Lajtha, 1973; Bender, 1975; Oldendorf & Szabo, 1976).

At the blood brain barrier, there are three known transport mechanisms which mediate the transport of neutral, acidic, and basic amino acids. Neutral amino acid transport systems may be subdivided into the L-system (leucine-preferring) and A-system (alanine-preferring). The L-system transports mainly large neutral amino acids, including leucine, isoleucine, tyrosine, phenylalanine, tryptophan and glutamine. The L-system is predominantly a facilitated carrier transport system which requires no energy or the presence of sodium. The functional features of this facilitated transport system is downhill diffusion, competitive transport, and exchange diffusion (Cohen & Lajtha, 1972; Rapoport, 1976). The A-system transports mainly small amino acids including alanine, serine, threonine and glycine. This system can maintain steep concentration gradients, and requires sodium for its operation. The plasma concentation of an amino acid is generally of the same order of magnitude as the binding constant for its transport into brain, indicating that the rate of transport will be very sensitive to changes in plasma concentration of either that amino acid or amino acids that compete with it for

transport. Such an equivalence of plasma amino acid concentration and binding constant is not found in other organs (Cutler, 1980). The A system has been shown to be located on the antiluminal side and its function is the outward transport. The combined effects of these two systems are the bidirectional transport of amino acids across the cerebral capillary (Cutler, 1980).

The brain cells, the neurons and astroglial cells have been shown to be able to actively take up amino acids from the extracellular space. On incubation, brain slices accumulate all amino acids against a concentration gradient until the quantity in the tissue is several times greater than endogenous levels (Levi et al, 1967; Archer & Breakefield, 1974; Bauman et al, 1974; Rapoport, 1976). Such uptake is found to be saturable and stereospicific. Hamberger (1971) convincingly demonstrated the active uptake of amino acids by neuronal and glial cells from rabbit cerebral cortex using radio-labeled amino acids. The total uptake rate, expressed as the radioactivity ratio between the cells and the medium ranged from 1.2 to 2.6 in the neurons, and from 1.4 to 71.5 in the glial cells. The accumulation of free amino acids in the neurons and glial cells was inhibited by ouabain and dinitrophenol. These experiments clearly indicate the active and energy-dependent nature of the amino acid transport mechanism on the cell membranes of the neurons and glial cells (Hamberger, 1971; Cutler, 1980; Newsholme & Leech, 1983).

Cerebrospinal fluid is sometimes viewed as part of the extracellular fluid in the brain. Evidence has been accumulated to show that extracellular fluid of the brain is in diffusion equilibrium with CSF.

There are no lymphatics in central nervous tissue. The proximity of nervous tissue to CSF provides a partial substitute for the lack of lymphatics. The total volume of CSF is relatively small, about 10% of the brain weight. It is in contact with central nervous tissue over considerable areas of the ependymal and pia-glial membranes (Bradbury, 1979). CSF flows over nervous tissue and allows the diffusion and removal of products of metabolism which could not easily pass into blood across the blood-brain barrier. Such a "sink" action of the CSF seems to occur for non-metabolized polar compounds of moderate molecular weights which penetrate the blood brain barrier slowly. Total amino acid concentration is only 30% as much as that in the plasma (Rapoport, 1976, Fishman, 1980). Thus this "sink" action is shown to play a role in the maintenance of brain extracellular amino acid concentration (Bradbury, 1979; Cutler, 1980). Neurotransmitter amino acids are known to be lost to the CSF (Cutler, 1980). The low concentration of amino acids is due to the active transport systems at the choroid plexus, and possibly, the subarachnoid spaces as well (Lajtha, 1962, 1973; Bender, 1975; Lorenzo et al, 1975). It has been demonstrated that the gamma-glutamyl cycle may be involved in actively transporting amino acids from CSF to blood (Bender, 1975).

All the factors discussed above need to be taken into account when analyzing the effects of hemoperfusion on the removal of amino acids from plasma, CSF and brain. Furthermore, since hemoperfusion was carried out in the rats with fulminant hepatic failure and coma, several other factors should be taken into account:

(1) Amino acids are markedly increased in plasma, CSF, brain, liver, kidney, skeletal muscle, and many other tissues (Record et al, 1976; Hughes et al, 1981; Holmin et al, 1983; Shi & Chang, 1984). Such a buildup of amino acids significantly increased the volume of distribution of most amino acids especially the aromatic amino acids.

(2) The blood brain barrier is found to be highly permeable or even severely disrupted in hepatic coma (Livingstone et al, 1977; Horowitz et al, 1983; Potvin et al, 1984; Zaki et al, 1984; Dixit & Chang, 1984). Due to the breakdown of the blood brain barrier, the interstitial fluid in the brain is more directly influenced by the plasma composition. The exchange and equilibrium of amino acids between the plasma and the interstitial fluid can now take place more readily and without the intenvention of the barrier.

(3) In hepatic coma, the brain cells, especially, the neurons, are found to be normal. The neuronal ultrastructure is found intact (Livingstone et al, 1977; Finlayson, 1982; Potvin et al, 1984). This observation may imply that the transfer of amino acids between the cells and the interstitial fluid is still under the influence of the amino acid transport

mechanisms which uptake and accumulate amino acids into the cells.

In evaluating the amino acid transfer in the brain compartments during and after the hemoperfusion, Gibson's 3-compartment model is found helpful (Gibson, 1980). This model includes (1) a plasma compartment; (2) a slow equilibrating compartment; and (3) a fast equilibrating compartment. This model has been used in illustrating the distribution of some drugs, and their removal by hemoperfusion. Gibson's model coincides with the 3 compartment model in the brain proposed by Rapoport (1976). Thus, the brain can be viewed as composed of a plasma compartment, a slow equilibrating compartment which includes the intracellular space in the neurons and the astroglial cells, and a fast equilibrating compartment which includes the interstitial fluid and the CSF.

The amino acid concentration in the brain reflects mostly the concentration in the cells since the intracellular fluid accounts for 80% of the water in the brain. Furthermore, the uptake mechanism makes the intracellular concentration of the amino acids much higher than the concentration in the interstitial space. The amino acids in the blood plasma are in rapid equilibrium with the interstitial fluid and CSF, but not as rapidly with the brain cells. Thus, the amino acid concentrations in the brain tissue are not noticeably affected by hemoperfusion even though CSF is significantly affected. Although it is speculated that the amino acid concentration in the interstitial fluid may be significantly decreased, such

change would not show when the brain tissue is measured as a whole without discriminating between the two compartments, the intracellular and the interstitial fluids.

The observation that aromatic amino acids are significantly reduced in CSF following 2 consecutive 1-hr activated charcoal hemoperfusion may be related to several factors. Firstly, the CSF is now formed from a plasma that has already been cleared of amino acids at a high clearance rate in hemoperfusion. Secondly, the CSF is now in equilibrium with an interstitial fluid which has been under the direct influence of plasma owing to the breakdown of the blood brain barrier. Thirdly, due to the relatively intact cellular membrane structures, the neuronal and, probably, also glial cells are not directly and immediately affected by the changes in extracellular and plasma amino acid concentrations.

4.3. MIDDLE MOLECULAR SUBSTANCES IN BLOOD AND BRAIN IN GALN-FHF RATS

4.3.1. Middle molecules in FHF rats: Their significance and the interrelationship between blood and brain

In fulminant hepatic failure, the disturbances in hepatic metabolism or products of cell necrosis, result in the accumulation of a large spectrum of unwanted metabolites. Molecules in the medium size range (500-2,000 daltons) have not been studied adequately.

In this thesis, the accumulation of middle molecular substances in blood and brain compartments were analyzed.

Using Sephadex G-15 gel permeation chromatographic separation and UV 206 nm absorbance tracing, the abnormal middle molecule peak 7 was shown to be elevated in the plasma of the FHF rats 48 hours after galactosamine injection. In normal rat plasma, no peak 7 was recorded. This result was in good accordance with the earlier studies from this laboratory (Chang & Lister 1982; Shu & Chang 1983).

The elution volume of the elevated peak 7 coincided with that of molecular size marker Vit.B12 (MW 1,355) indicating the approximate molecular size of the middle molecules. As the separable molecular weight range is below 1,500 daltons in the Sephadex G-15 molecular size exclusion chramatography, the molecules in the peak 7 fraction can be estimated in the vicinity of 1,355, or, roughly 1,000-1,500 daltons.

When the peak 7 fractions from the gel permeation chromatoraphy were further analyzed using anion-exchange gradient elution, the middle molecular subpeaks $7c_2$, 7d and 7g in FHF rat plasma were found elevated by 1.6 to 3.3 folds compared to the normal plasma samples.

Taken as a whole, peak 7 was not significantly increased in the brain of FHF rats. However, when peak7 was subdivided into its subpeaks in the ion-exchange elution, significant changes were observed. The PHU values of the subpeaks 7d and 7g in FHF rat brain were significantly elevated by 1.8 to 1.9 fold. The accumulation of middle molecules in the brain, in so far as the subpeaks were concerned, appeared to be parallel to the increases in the plasma MM levels in the GalN-FHF rats.

The concomitant increase in the middle molecule levels in both the plasma and the brain implies the probable peripheral origin of the brain middle molecules. There has been ample evidence indicating the increased permeability and even a destruction of the blood brain barrier in fulminant hepatic failure (Livingstone et al, 1977; Horowitz et al, 1983; Zaki et al, 1983; 1984). These studies demonstrated that the blood brain barrier became highly permeable to some substances which should otherwise be excluded by a normal blood brain barrier. Among the substances tested, it is noteworthy that the blood brain barrier became permeable even to large molecules such as inulin (MW 3,000) and trypan blue (MW 961). In another study, an increased permeability of the blood brain barrier for medium-sized hormonal polypeptide, angiotensin I (MW 1,290), was demonstrated in rats with ischemic liver injury (Crinquette & Boschat, 1982). The breakdown of the blood brain barrier was believed to be caused by the circulating toxic metabolites such as phenols, mercaptans and fatty acids which accumulate in the blood due to the inefficiency of the liver in metabolizing these toxic substances (Zaki et al,1984). The breakdown of the blood brain barrier has been suggested to be due to: (1) the opening of intercellular tight junctions in the capillary wall (Horowitz et al, 1983); and (2) the increased number of vesicles within cerebral endothelial cells which mediate the augmented transfer of those otherwise nonpermeable molecules (Laursen & Westergaard, 1977; Potvin et al, 1984).

These pathohistological findings are likely to account for the observed middle molecule increase in the brain. The disrupted blood brain barrier in FHF no longer constitute a restraint to the passage of the middle molecular substances into the brain.

After ACAC hemoperfusion treatment, the significant reduction of subpeak 7g in the plasma was followed by a reduction of 7g in the brain. Other subpeaks $7c_1$, $7c_2$ were decreased in the plasma but not in the brain. The significant decrease of subpeak 7g in the brain following the decrease of 7g in the plasma is probably due to the following reasons:

(1) The high clearance rate of activated charcoal in hemoperfusion: Activated charcoal has proven to be very effective in removing medium-sized molecules, especially peptide substances in hemoperfusion (Chang & Migchelsen, 1973; Chang et al, 1974; Oules et al, 1977; Leber et al, 1981; Nishiki et al, 1984; Kawanishi et al, 1984).

(2) The increased permeability of the blood brain barrier in fulminant hepatic failure: This permeability is either due to the opening of the intercellular tight junction (Horowitz et al, 1983) or the increased number of transport vesicles in the cerebral capillary endothelial cells (Laursen & Westergaard, 1977; Potvin et al, 1984).

(3) A probable small volume of distribution: Since these middle molecule metabolites are large and water soluble (proven by the aqueous buffer extraction procedures before column chromatographic analyses), they are probably unable to

penetrate the cell membranes without special transport mechanism. This may mean that these middle molecule substances are present mainly in the extracellular spaces.

The fact that the contents from subpeak 7g fraction substance in the brain rise and fall concurrently with the subpeak 7g substances in the plasma suggests the dependence of these middle molecular metabolites on the extracellular space. This will further support the postulation that the subpeak 7g middle molecules have a small volume of distribution which is mainly within the blood and the interstitial fluid.

It is therefore probable that the decrease in middle molecules in the brain after hemoperfusion was due to: (1) a high clearance rate of middle molecules from plasma by activated charcoal hemoperfusion; (2) the breakdown of the blood brain barrier; and (3) a suggested small volume of distribution of the middle molecules.

As discussed earlier, the interstitial fluids being in rapid equilibrium with the blood plasma are classified as the fast equilibrating compartment. On the other hand, intracellular fluids represent the slow equilibrating compartment since most tissue cells display the capability of actively transporting and accumulating amino acids against a concentration gradient. In fact, it is the intracellular amino acid concentrations that determine the total tissue amino acid content, since the intracellular fluid in most cases accounts for the overwhelming majority of the tissue wet weight. There is, however, some heterogeneity among different tissues in

their rates of amino acid transfer between the interstitial and intracellular spaces. The liver, for example, has an interstitial space which is immediately accessible to substances present in the circulating blood. The unique structure of the sinusoids and the hepatic cell plates is such that diffusion equilibration of substances beween the extravascular space and the intracellular space would occur much more rapidly (Goresky, 1969). The histological structure of the liver probably favors a relatively faster equilibrium of the amino acids with the interstitial space than do the other tissues, including brain and muscle. However, owing to the transport mechanisms that control the uptake and efflux of amino acids across the cell membranes, the tissue cells are generally slower than the interstitial fluid in response to the amino acid changes in the plasma.

4.3.2. Preliminary characterization of middle molecules in fulminant hepatic failure

Although the methodologies used here were originated from the techniques in uremic middle molecule analyses, the components of the abnormal subpeaks in FHF were not the same as in uremia. In uremic patients, it was subpeak 7d that was significantly elevated and correlated well with uremic symptoms. However, in FHF it was subpeaks $7c_2$, 7d and 7g in plasma, and 7d and 7g in brain that correlated with the course of hepatic coma.

Furthermore, after hemoperfusion it was 7g that responded, in both blood and brain compartments, to the adsorption effects of charcoal. Therefore subpeak 7c in uremia and subpeak 7g in FHF are two distinct types of substances. They differ from each other in their chromatographic behaviour in anion-exchange gradient-elution, although they may have similar molecular size which fall into the fractional coverage of the middle molecular peak 7 in Sephadex gel-permeation. The molecular weight of hepatic failure midle molecule subpeak 7 fraction was estimated at the vicinity of 1,300 dalton. The molecular weight range of peak 7 may be estimated from 500 to 1,500. The upper limit border line is the molecular cut-off of the Sephedex G-15 gel.

The chemical identity of hepatic failure middle molecules heve been studied by a number of centers. Leber et al found the existence of ninhydrin-positive middle molecules in the gel permeation chromatographic fractions derived from cirrhotic patients, from Amanita phalloides intoxicated patients, and from galactosamine induced hepatic failure rats (Leber et al, 1981). Opolon described the finding of fluorescent substances with a molecular weight over 1,000 but below 4,000 in plasma and brain of pigs with acute liver ischemia and hepatic coma (Opolon, 1980). Seda et al found that serum from patients with fulminant hepatic failure inhibits rat brain Na+, K+-ATPase activity in vitro. Such inhibition existed in the G-25 gel permeation fractions including middle molecular substances (Seda et al, 1984).

In my study, protein-dye-positive substances in the middle molecular subpeak 7g fraction were demonstrated. In view of the molecular weight range of peak 7 (500-1,500), the protein-dye-positive substances in 7g should be logically considered to be peptides. In SDS-PAGE electrophoretic analysis of FHF rat plasma, the revelation of peptidic bands strongly suggested the peptidic nature of some constituents of the middle molecules in hepatic failure. Furthermore, based on the calculations from the molecular weight standards, which were derived from the partial cleavage of sperm whale myoglobin, the molecular weights of the abnormal peptides in hepatic failure were estimated to be 1,300 to 1,450 dalton. This estimated molecular weight and the SDS-PAGE revealed peptides are believed to be the first report on the hepatic failure-related middle molecules. Further studies have been carried out using radio-immunoassays to determine whether or not known neuropeptides were involved in the developement and the course of hepatic failure. Beta-endorphin concentrations in FHF plasma and brain were found unchanged when rats were sacrificed without anesthesia. The observation of elevation of substance P level in FHF rat plasma is an interesting evidence of the possible involvement of this neuropeptide in the course of hepatic failure. Substance P is a putative peptidic neurotransmitter which has numerous actions on peripheral tissues, including contraction of smooth muscle in the gut and urinogenital tract; lowering of blood pressure; increasing capillary permeability; releasing histamine from mast cells;

and secretagogue activity in the pancreas and salivery glands (Iversen 1982). It has also been observed that, substance P has an analgesic effect in mice and rats after central or parenteral administration (Skrabanek et al, 1980). Observations in humans also confirmed the analgesic effect of Substance P.

The main source of substance Pin plasma is believed to be the intestines. Gamse et al reported that in the cat the major part of circulating SP originates from the intestine. SP released from the intestine then traverses through the portal vein and reaches the liver where it is inactivated and degraded (Gamse et al, 1978). Therefore, human SP levels in the portal vein are higher than in the hepatic vein.

Liver is an important site of substance P degradation in vivo (Skrabanek & Powell, 1980). This has been confirmed by many experiments in which substance P was less biologically potent following portal vein infusions than following peripheral vein infusions. In rats, Lembeck et al observed that 90% of infused substance P (as measured by salivation response) was destroyed by passage through the liver when SP(1 ug) was infused into the portal vein as compared with the infusion of the same amount into the jugular vein (Lembeck et al, 1978).

Therefore, in hepatic failure, circulating SP accumulates as a result of deficient degradation of the peptide during transhepatic passage. Hörtnagl H. et al recently reported the marked increase of SP in plasma of 18 patients with hepatic

coma(stage 1-4) (Hörtnagl et al, 1984).

Because of the strong analgesic effect (Skrabanek et al, 1980; Olsson et al, 1977), and the potent vasodilating properties of SP (Eklund 1977), the accumulation of SP may contribute to the composition and the neurotoxic effects of the middle molecules in hepatic failure. Besides, the consequential increase of capillary permeability (Iversen, 1982) may also contribute to the malfunction of the blood brain barrier.

4.4. Summary of the removal of amino acids and middle molecules by hemoperfusion

The removal of amino acids and middle molecules from the plasma and the responses of the CSF and brain in hemoperfusion are summarized here.

(1) In the hemoperfusion studies for amino acids removal, the animal body is considered to be composed of 3 compartments which are plasma, interstitial fluid and intracellular fluid. Aromatic amino acids are significantly reduced in the plasma by charcoal adsorption or artificial cell hemoperfusion. CSF aromatic amino acids were significantly reduced following the 2 consecutive 1-hr CAC hemoperfusion. Brain aromatic amino acids were not significantly affected by the reduced plasma and CSF amino acid concentrations.

A high plasma clearance removes a considerable amount of aromatic amino acids. The breakdown of the blood brain barrier favors a closer and more direct equilibrium between the plasma

and the interstitial fluid. CSF is formed directly from a plasma that is cleared of aromatic amino acids at appreciable amounts. CSF is now in equilibrium with an interstitial fluid that is directly under the influence of plasma composition with reduced levels of aromatic amino acids. Furthermore, the "sink" effects produced by the active transport mechanisms at the choroid plexus may add to the net reduction of aromatic amino acids in CSF. In the intracellular compartment, however, the relatively intact cellular structure has been maintained and the amino acids are still being under the close control of the active transport mechanisms. Thus, the amino acid concentrations in the brain tissue as a whole (containing 80% of intracellular components) are not significantly influenced by plasma amino acid removal in the hemoperfusions using activated charcoal or tyrosinase artificial cells.

(2) In the hemoperfusion study for middle molecule removal, the breakdown of the blood brain barrier causes the penetration of the middle molecule subpeak 7g substances into the brain extracellular space. These medium-sized (MW 500-2,000) abnormal metabolites are probably unable to penetrate the cell membranes due to their large size and hydrophillic nature. The significant reduction in brain subpeak 7g in response to the plasma reduction is likely an indication that subpeak 7g middle molecules exist mainly in the extracellular fluid compartment, i.e., in the circulation and the interstitial fluid.

CHAPTER 5.

SUMMARY AND CLAIMS TO ORIGINAL RESEARCH

(The * marks denote the claims to the original research)

5.1. Acute massive hepatocyte necrosis and fulminant hepatic failure is produced by intraperitoneal injection of the selective hepatotoxin galactosamine. Electron microscopic examination revealed the hepatocyte ultrastructural changes which resemble that of human fulminant hepatic failure.

5.2.* Galactosamine induced fulminant hepatic failure produced a severe state of catabolism and negative nitrogen balance. This is characterized by a significant decrease of body weight and a significant reduction in plasma albumin and total protein concentrations.

5.3.* In galactosamine induced FHF rats, most amino acids were found significantly increased in systemic blood plasma, portal plasma, CSF, cerebrum, liver, kidney and skeletal muscle amino acid pools.

5.4.* The free amino acid pool in the skeletal muscle constitutes the greatest source of accumulated amino acids in FHF. The other tissues also contribute to the increased amino acids in the body as a whole in GalN-FHF.

5.5.* The increase in concentrations of the aromatic amino acids tyrosine, phenylalanine, and free and total tryptophan were the most striking among all the amino acids in all the tissues studied. The molar ratio of BCAA/AAA was found significantly reduced in all the tissues studied.

5.6.* Gamma-amino butyric acid was found significantly increased in the cerebrum and the brain stem in GalN

induced FHF rats.

5.7.* Tryptophan was significantly increased in plasma, CSF, brain, liver, kidney and skeletal muscle. Further analysis showed that most of increased tryptophan in plasma, and almost 100% of the increased tryptophan in the brain were in the free form.

5.8.* The increase in tyrosine concentration in plasma was correlated with tyrosine in the brain or in the liver.

5.9.* In vitro adsorption spectrum of amino acids by collodion coated activated charcoal displayed a preferential removal of aromatic amino acids over other amino acids including the branched chain amino acids. The magnitude of removal was found to be related to the hydrophobicity of the side groups of the amino acids.

5.10.*Two consecutive 1-hr hemoperfusions with collodion coated activated charcoal in grade 3 hepatic coma rats substantially reduced levels of aromatic amino acids in the plasma. This was followed by a reduction in aromatic amino acids in the CSF, but not in the brain.

5.11.*Hemoperfusion with tyrosinase immobilized within artificial cells selectively reduced tyrosine level in the plasma in GalN-FHF rats. The brain level of tyrosine was not affected by tyrosinase artificial cell hemoperfusion.

5.12.*Detailed methodologies were worked out for amino acid analysis in physiological samples including plasma, CSF

and tissue extracts using reverse phase high performance liquid chromatography (HPLC).

5.13.*The existence of peptidic substances was demonstrated in the middle molecule fractions separated from the plasma of GalN-FHF rats by serial liquid chromatographies of Sephadex gel permeation and anion exchange elution.

5.14.*Hemoperfusion with collodion coated activated charcoal significantly reduced the levels of some middle molecule components in the fraction of anion exchange chromatographic subpeak 7g. The significant reduction in 7g components was found in both plasma and brain extract samples from hemoperfused rats with grade 3 hepatic coma.

5.15.*Preliminary attempts have been made to characterize the middle molecular substances in GalN-FHF rats:

(1) SDS-polyacrylamide electrophoresis has revealed the existence of peptides in the plasma extracts from GalN-FHF rats. The molecular weight of the middle molecule peptides was estimated to be 1,300 to 1,400 daltons as calculated from the peptide standards.

(2) Radio-immunoassay of beta-endorphin showed that after anesthesia, the beta-endorphin level in GalN-FHF rat plasma was significantly lower than that in the normal rats plasma.

(3) Immuno-reactive substance P was found significantly increased in the plasma of GalN-FHF rats in comparison to the normal subjects.

BIBLIOGRAPHY

Abiko T, Onodera I, Seikino H: Isolation, structure and biological activity of the Trp-containing pentapeptide from uremic fluid. Biochem Biophy Res Commun 89:813-821, 1979.

Abouna GM, Barabas AZ, Alexander F, Boyd N, Todd JK, Kinniburgh DW, Gilchrist T: Animal models of hepatic failure for evaluation of artifical liver support techniques. in: Kenedi RM, Courtney JM, Gaylor JDS, Gilchrist TG (eds) Artificial organs, MacMillan: London, p 351-362, 1976.

Abouna GM, Barabas AZ, Boyd N, Todd JK, Alexander F, Kinniburgh DW, Gilchrist T, Jonsson E: Resin and charcoal hemoperfusion in the treatment of hepatic coma. In: Kenedi TM, Courtney JM, Gaylor JDS, Gilchrist T (eds) Artificial Organs. London: MacMillan Press, 363,1977.

Abouna GM, Gilchrist T, Pettit JE, Boyd ND, Todd JK, Courtney JM & Maini R: Hemoperfusion with activated charcoal in treatment of experimental acute hepatic failure. In: Williams R & Murray-Lyon IM (eds) Artificial Liver Support, Kent: Pitman Medical, 180-185,1974.

Adams F: The genius works of hippocrate. Baltimore, Williams & Wilkins, 58, 1939

Agishi T, Yamashita N & Ota K: Clinical results of direct charcoal hemoperfusion for endogenous and exogenous intoxication including hepatic failure. In: Sideman S, Chang TMS (eds) Hemoperfusion: kidney and liver support and detoxification. Hemisphere: Washington, p 255-265, 1980.

Amano I, Kano H, Takahira H, Yamamoto Y, Itoh K, Iwatsuki S, Maeda K & Ohta K: Hepatic assist system using bead-type charcoal. In: Chang TMS (ed) Artificial Kidney, Artificial Liver, and Artificial Cells. New York: Plenum Press, p 89, 1978.

Amatuzio DS, Nesbitt S: A study of pyruvic acid in the blood, spinal fluid and urine of patients with liver disease with and without hepatic coma. J Clin Invest, 29: 796, 1950.

Anastacio M, Hoyumpa PVD Jr, Avant GR, Roderick KR Shenker S: Hepatic encephalopathy. Gastroenterology, 76: 184-195, 1979.

Andrade JD, Kunitomo K, Van Wagenen R, Kastigir B, Gough D & Kolff WJ: Coated adsorbents for direct blood perfusion: HEMA/activated charcoal. Trans Am Soc Artif Intern Organs 17: 222-228, 1971.

Archer EG & Breakefield X: Transport of tyrosine and phenylalanine in cultural neuroblastoma cells. Trans Am Soc Neurochem, 5: 98, 1974.

Babb AL, Popovich RP, Christopher TG & Scribner BH: The genesis of the square meter-hour hypothesis. Trans Am Soc Artif Int Organs, 17: 81, 1971.

Babb AL, Farrell PC, Urelli DA & Scribner BH: Hemodialyzer evaluation by examination of solute molecular spectra. Trans Am Soc Artif Int Organs, 18: 98, 1972.

Balo J, Korpassy B: Encephalitis of dogs with Eck fisula fed on meat diets. Arch Path, 13: 80, 1932.

Bandel W: Worksess. 7th working group clin. Nephrol Hamburg, 1974.

Barman TE: In: Enzyme Handbook. Spring-Verlag, New York, Vol 1: 226, 1969.

Barnes JM & Magee PN: Some toxic properties of dimethylnitrosamine. Br J Ind Med, 11: 167, 1954.

Bartels O: Hemoperfusion through activated carbon adsorbents in liver-failure and hepatic-coma. Act Hep Gas, 25: 324, 1978

Bass NH & Lundborg P: Transport mechanisms in the cerebrospinal fuid system for removal of acid metabolites from developing brain. In: Levi G, Battistin L & Lajtha A (eds) Transport phenomena in the nervous system. Plenum Press, New York, London, p 31, 1975.

Battersby C, Hickman R, Saunders SJ, Terblanche J: Liver function in the pig: 1. The effects of 30 minutes normothermic ischaemia. Br J Surg, 61: 27-32, 1974.

Bauman A, Bourgoin S, Benda P, Glowinski J & Hamon M: Characteristics of tryptophan accumulation by glial cells. Brain Res. 66: 253-263, 1974.

Bayer E, Grom E, Kaltenegger B & Uhman R: Separation of amino acids by high performance liquid chromatography. Anal Chem, 48: 1106-1109, 1976.

Bender DA: Amino acid metabolism. John Wiley & Sons, London, p 183, 1975.

Bernelli ZA: Endogenous inhibitors of protein synthesis in ischaemic livers. In: Dianzani MU, Ugazio G, Sena LM (eds) Recent advances in biochemical pathology of toxic liver injury, Minerva Mdica, p 45, 1975.

Berk PD & Popper H: Fulminant hepatic failure. Am J Gastroenterology 9(3): 349-400, 1978.

Bessman SP: Ammoniagenic coma: The chemistry of an endogenous intoxication. Proc 4th Int Cong Biochem, Vienna, p 141-145, 1958.

Blasberg R, Lajtha A: Heterogeneity of the mediated transport systems of amino acid uptake in brain. Brain Res, 1: 86-104, 1966.

Blitzer BL, Waggoner JG, Jones EA, Gramick HR, Towme D, Butler J, Weise V, Kopin IJ, Walters I, Teychenne PF, Goodman DG & Berk PD: A model of fulminant hepatic failure in the rabbit. Gastroenterology, 74: 664-671, 1978.

Blume U, Baldamus G, Schmidt E, Hemstaedt D, Sussman P, Schmidt FW, Sybrecht G & Heyer U: Hemoperfusion therapy for acute hepatic failure. Deutsch Med Wochen, 101: 559, 1976.

Bollmam JL & Mann FC: Experimentally produced lesions of the liver. Annals Intern Med, 5: 699-712, 1931-32.

Bollman JL, Flock EV, Grindlay JH, Bickford RG, Lichtenheld FR: Coma with increased amino acids of brain and cerebrospinal fluid in dogs with Eck's fistula. Arch Surg. 75: 405-412, 1975.

Bollman JL & van Hook E: A simplified two-stage hepatectomy in the rat. J Applied Physiol, 24(5): 722-723, 1968.

Bright R: Observations on Jaundice. Guy Hosp Rep, 1: 604, 1836. Quoted from Brown I: Liver brain relationships. Springfield, Thomas, p 9, 1957.

Brown H: Hepatic failure. Springfield, Thomas, p 7, 1970.

Brown IA: Liver-brain relationships. Springfield: Thomas, p 7, 1957a.

Brown IA: Ibid, p 19, 1957b.

Brun A, Dawiskiba S, Hindfelt B & Olson J-E: Brain proteins in hepatic encephalopathy. Acta Neurol Scand 55:213-225, 1977.

Brunner G, Windus G & Lösgen H: On the role of free phenols in the blood of patients in hepatic failure. In: Brunner G, Schmidt FW (eds) Artificial Liver Support Berlin Heidelberg: Springer-Verlag, p 25-31, 1981.

Cameron GR & Karunaratre WAE: Carbontetrachloride cirrhosis in relation to liver regeneration. J Path Bact 42(1): 1-21, 1936

Carpi A: The classification of drugs acting on cerebral circulation. in: Carpi A (ed) Pharmacology of the cerabral circulation, International encyclopedia of pharmacology and therapeutics, Vol 1(33), p 87, 1972.

Cardelli-Cangiano P, Cangiano C, James H, Jeppsson B, Brenner W & Fischer JE: Uptake of amino acids by brain microvessels isolated from rats after portacaval anastomosis. J Neurochem 36(2): 627-632, 1981.

Caster WO, Poncelet J, Simon AB,& Armstrong WD: Tissue weights of the rat, I. Normal values determined by dissection and chemical methods. Proc Soc Exp Biol Med 91: 122-126, 1956.

Chang TMS: Hemoglobin corpuscles: Report of a research project for the B.SC. Honours, McGill University, Montreal, 1957.

Chang TMS: Semipermeable microcapsules. Science, 146: 524, 1964.

Chang TMS: Semipermeable aqueous microcapsules (artificial cells): with emphasis on experiments in an extracorporeal shunt system. Trans Am Soc Artif Intern Organs, 12: 13, 1966.

Chang TMS: Removal of endogenous and exogenous toxins by microencapsulated absorbent. Can J Physiol Pharmacol, 47(12): 1043, 1969

Chang TMS: Artificial cells. Charles C Thomas: Springfield, 1972a.

Chang TMS: Hemoperfusion over microcapsulated adsorbent in a patient with hepatic coma. Lancet, 2: 1371, 1972b.

Chang TMS & Migchelsen M: Characterization of possible "toxic" metabolites in uremia and hepatic coma based on the clearance spectrum for larger molecules by the ACAC microcapsule artificial kidney. Trans Am Soc Artif Intern Organs, 19: 314, 1973.

Chang TMS, Experience with the treatment of acute liver failure patients by hemoperfusion over biocompatible microencapsulated (coated) charcoal. In: Williams R & Murray-Lyon IM (eds) Artificial Liver Support, Pitman, London, p 229-233, 1975.

Chang TMS, Lister C, Chirito E, O'Keefe P & Resurreccion E: Effects of hemoperfusion rate and time of initiation of ACAC charcoal hemoperfusion on the survival of fulminant hepatic failure rats. Trans. Am. Soc. Artif. Intern. Organs, 24: 243, 1978.

Chang TMS & Lister C: Analysis of possible toxins in hepatic coma including the removal of mercaptan by albumin-collodion charcoal. Int J Artif Organs, 3(2): 108-112, 1980.

Chang TMS & Lister C: Middle molecules in hepatic coma and uremia. Artif Organs, 4(supp): 169-172, 1981.

Chang TMS: Hemoperfusion, exchange transfusion, cross circulation, liver perfusion, hormones and immobilized enzymes. In: Brunner G & Schmidt FW (Eds) Artificial Liver Support. Springer-Verlag, Berlin, pp 126-133, 1981.

Chang TMS, Barre P, Kuruvilla S, Man NK, Lacaille Y, Messier D, Messier M & Resurreccion E: Hemoperfusion-hemodialysis in a single unit: Composite artificial kidney. Artificial support systems, 1: 63-67, 1982.

Chang TMS: Liver support systems. In: Bartlett R, Whitehouse WM & Turcotte JG (Eds) Life Support Systems in Intensive Care, Year Book Publisher, Chapter 18, p 461-485, 1984a.

Chang TMS: Coated charcoal hemoperfusion. Life Support Systems, 2: 99-106, 1984b

Chang TMS: Microencapsulation and artificial cells. Humana Press, 1984c.

Chapman GV, Ward RA & Farrell PC: Separation and Quantification of the "middle molecules" in uremia. Kidney Int, 17: 82, 1980.

Chase RA, Pavies M, Trewby PN, Silk DBA & Williams R: Plasma amino acid profiles in patients with fulminant hrpatic failure treated by repeated polyacrylonitrile membrane hemodialysis. Gastroenterology 75(6): 1033-1040, 1978.

Cheldelin VH & Williams RJ: J Am Chem Soc, 64: 1513, (1942), cited from: Greenstein & Winitz (eds), Chemistry of the amino acid. Vol II, John Wiley & Sons Inc, p 1448, 1961.

Chen S, Mahadevan V & Zieve L: Volatile fatty acids in the breath of patients with cirrhosis of the liver. J Lab Clin Medicine 75: 622-627, 1970.

Chesney AM, Marshall EK & Rowntree LG: Studies in liver function. JAMA 63: 1533, 1914.

Chirito E, Reiter B, Lister C & Chang TMS: Artificial liver: the effect of ACAC microencapsulated charcoal hemoperfusion on fulminant hepatic failure. Artif Organs, 1: 76, 1977.

Chirito E, Lister C & Chang TMS: Biochemical-hematological and histological changes in a fulminant hepatic failure rat model for artificial liver assessment. Artificial Organs, 3(1): 42, 1978.

Chirito E, Reiter B, Lister C & Chang TMS: Assessments of two rat models of fulminant hepatic failure for testing artificial liver devices. In: Chang TMS (ed) Artificial Kidney, Artificial Liver and Artificial Cells. New York: London, Plenum Press, 239-243, 1978.

Chojkier M & Fierer J: D-Galactosamine hepatotoxicity is associated with endotoxin sensitivity and mediated by lymphoreticular cells in mice. Gastroenterology 88:115-121, 1985

Christensen HN, Streicher JA & Elbinger RL: Effects of feeding individual amino acids upon the distribution of other amino acids between cells and extracellular fluid. J Biol Chem, 172: 515-524, 1948

Christensen HN: Mode of transport of amino acids into cells. In: McElroy WD & Glass HB (eds) Amino acid metabolism. Baltimore, Johns Hopkins Press, p 63-106, 1955.

Christensen HN: Relevance of transport across the plasma membrane to the interpretation of the plasma amino acid pattern. In: Leathem JH (ed) Protein nutrition and free amino acid patterns, Rutgers University Press: New Brunswick, p 407, 1965.

Christensen HN: Developments in amino acid transport illustrated for the blood brain barrier. Biochemarmacol, 28: 1989-1992, 1979.

Cichocki T, Hanicki Z, Klein A, Konorowska Z, Sarnecka-Keller M & Sulowicz W: Influence of middle-molecule-weight solutes from dialysate on the migration rate of leucocytes. Kidney Int, 17: 231-236, 1980.

Cohen SR & Lajtha A: Amino acid transport. In: Lajtha A (ed) Handbook of neurochemistry, Plenum Press, New York, Vol 7, p 543-572, 1972.

Condon RE: Effect of dietary protein on symptoms and survival in dogs with an Eck fistula. Am J Surg, 121: 107-114, 1971

Coulson RA, Hernandez T & Herbert JD: Factors affecting amino acid transport in vivo. In: Silbernagl S, Lang F & Greger R (eds) Amino acid transport and uric acid transport. Georg Thiem Publishers, Stuttgart, p 119, 1974.

Cowen PJ & Nutt DJ: Abstinence symptoms after withdrawal of tranquillizer drug: is there a common neurochemical metabolism? Lancet 2: 360-362, 1982.

Crinquette JF, Boschat M, Rapin JR, Delorme ML & Opolon P: Early changes in blood-brain barrier permeability after portocaval shunt (PCS) and liver ischaemia. Clin Physiol, 2(3): 241-250, 1982.

Cueille G, Man NK, Sausse A, Farges JP & Funck-Brentano JL: Further characterization of a neurotoxic uremic molecule. Proc 8th Int Congr Nephrol, Athens p 606-617, 1981.

Cuillert G, Pomier-Layrargues G, Pons F, Cadilhac J & Michel H: Changes in brain catecholamine levels in human cirrhotic hepatic encephalopathy. Gut 21: 565, 1980.

Curzon G & Knott PJ: Enviromental, toxicological and related aspects of tryptophan metabolism with particular reference to the central nervous system. Critical Review in Toxicology. 5: 145, 1977.

Curzon G & Knott PJ: Fatty acids and the disposition of tryptophan. In: Aromatic amino acids in the brain. Ciba Foundation symposium 22 (new series). Elsevier:Amsterdam, p 217-229, 1974.

Cutler RWP: Neurochemical aspects of blood-brain-cerebrospinal fluid barriers. In: Wood JH (ed) Neurobiology of cerebrosipinal fluid, Chapter 4, Plenum Press, New York, p 45, 1980.

Czygan P, Walker S, Sieg A, Stiehl A, Lanzinger G & Kommerell B: Effect of liver adapted amino acids on portosystemic encephalopathy in patients with cirrhosis of the liver and portasystemic shunts. In Brunner G & Schmidt (eds) Artificial liver support, Springer-Verlag, Berlin, p 170-171, 1981.

Dall'Aglio P, Buzio C. Cambi V, Arisi L & Migone L: La retention des moyennes molecules dans le serum uremique. Proc Eur Dial Transplant Ass, 9: 409-415, 1972.

Davidson CS: Pathophysiology of liver. In: MacSween RNM, Anthony PP, Scheuer PJ, Churchill Livingstone (eds) Pathology of the liver. Edinburgh: London: New York, p 32, 1979.

Decker K & Keppler D: Galactosamine induced liver injury. In: Popper H & Schaffner F (eds) Progress In Liver Disease. Grune & Stratton, New York, Vol 4, Chap 11, 1972.

Decker K, Keppler D, Rudigier J & Domschke W: Cell damage by trapping of biosynthetic intermediates. Hoppeseyler's Z Physiol Chem, 352: 412-418, 1971.

Dedrick RL & Bischoff KB: Pharmacokinetics in applications of the artificial kidney.Chem Eng Progr Sym Ser, 84(64): 32,1968.

Degos F, Degos J-D, Bourdiau D, Peignoux M, Prandi D, Rochesicot J, Sicot C, Rueff B & Benhamou J-P: Experimental acute hepatic encephalopathy: Comparison of the electroencephalograpgic changes in the liverless and in the eviscerated rat. Clin Sci Molecul Med 47: 599-608, 1974.

Denis J, Opolon P, Nusinovici V, Granger A & Darnis F: Treatment of encephalopathy during fulminant hepatic failure by hemodialysis with high permeability membrane. Gut 19: 787-793, 1978.

Denti E, Luboz MP & Tessorc V: Adsorption characteristics of cellulose acetate coated charcoal. J Biomed Mater Res, 9: 143-150, 1975.

DeVico D.C. Reye's Syndrome: a metabolic response to an acute mitcchondrial insult? Neurology (Minneap), 28, 105-108, 1978.

Dianzani MU & Gravela E: Inhibition of protein synthesis in carbon trtrachloride-induced liver injury. In: Keppler D (ed) pathogenesis and mechanisms of liver cell necrosis, University Park Press, Baltimore, 1974.

Dixit V & Chang TMS: Soluble liver extracts as supplement to detoxification by hemoperfusion. Int J Artif Organs, 4: 291-294, 1981.

Doizaki WM & Zieve L: An improved method for measuring blood mercaptans. J Lab Clin Med, 90: 849-855, 1977.

Duffy TE, & Plum F: Hepatic encephalopathy. In: Arias I, Popper H, Schachter D & Shafritz DA (eds) The liver: Biology and pathobiology, Raven Press, New York, Chapt 41, p 693-715, 1982.

Dunea G & Kolff WJ: Clinical experiments with the Yatzidis charcoal artificial kidney. Trans Am Soc Artif Intern Organs 11: 178-182, 1965.

Dzurik R: Hupkova Viera, Cernacek P, Valovicova E, Niederland TR: The isolation of an inhibitor of glucose utilization from the derum of uremic subjects. Clinica Chim Acta, 46: 77-83, 1973.

Eck NV: Concerning ligation of the vena porta. The Military Medical Journal, 130(2): 1-2, 1877. English translation cited by Child CG, In: Eck's fistula. SGO, 96: 375-376, 1953.

Eklund B, Jogestrand T & Pernow B: Effect of substance P on resistance and capacitance vessels in the human forebrain. In: von Euler US, Pernow B (eds) Substance P, Raven Press, New York, p 275-285, 1977.

Elliott TR & Walshe FMB: The babinski or extensor from of plantar response in toxic states. Lancet 1: 65, 1925.

El-Mofty SK, Scrutton MC, Serroni A, Nicolini C & Farber JL: Early reversible plasma membrane injury in galactosamine-induced liver cell death. Am J Pathol, 79(3): 579-593, 1975.

Engelhart H, Asshauer J, Neue U & Weigand N: Separation on heavily loaded small particle columns in high speed liquid chromatography. Anal Chem, 46: 336-340, 1974.

Enna SS: Regional variation and characteristics of GABA-receptors in the mammalian CNS. In Mandel P& DeFeudis FV (eds) BAGA biochemistry and CNS function, Plenum Press, New York, p 323-338, 1978.

Eriksson LS, Persson A & Wahren J: Branched-chain amino acids in the treatment of chronic hepatic encephalopathy. Gut, 23(10): 801-806, 1982.

Faguer P, Delorme ML, Dennis J, Cueille G, Boschat M & Opolon P: Demonstration of middle molecules in plasma and brain during experimental acute hepatic encephalopathy. Gastroenterology, 79: 1014, 1980.

Farrell PC & Skalsky M: Hemoperfusion for removal of protein-bound, lipid soluble drugs. In: Sideman S & Chang TMS (eds): Hemoperfusion, kidney and liver support and detoxification. Hemisphere Publishing Corp, Washington, New York, 1980.

Fenton JCB, Knight EJ & Humpherson PL: Milk and cheese diet in portal-systemic encephalopathy. Lancet 1: 164-165, 1966.

Ferenci P & Wewalka F: Plasma amino acids ir hepatic encephalopathy. J Neural Transmission, Suppl 14: 87-94, 1978.

Ferenci P, Covell D, Schafer DF, Waggoner JG, Shrager R, Anthony Jones E: Metabolism of inhibitory neurotransmitter gamma amino butyric acid in a rat model of fulminant hepatic failure. Hepatology, 3(4): 507-572, 1983.

Fernstrom JD & Wurtman RJ: Brain serotonin content: physiological regulation by plasma neutral amino acids. Science, 178: 414-416, 1972.

Finlayson M: Observations on the pathogenesis of hepatocerebral degeneration in cirrhosis. Bol Estud Med Biol, Mex 32: 3-11, 1982.

Fisher JE & Baldessarini RJ: False neurotransmitters and hepatic failure. Lancet, July 10(2): 75-79, 1971.

Fischer JE, Yoshimura N, Aguirre A, James JH, Cummings MG, Abel RM & Deindoerfer F: Plasma amino acids in patients with hepatic encephalopathy: effects of amino acid infusions. Am J Surg, 127: 40-47, 1974.

Fischer JE: On the occurence of false neurochemical transmitters. In: Williams R Murray-Lyon IM (eds) Artificial Liver Support, Kent: Pitman Medical, 31, 1974.

Fischer JE, Funovics JM, Aguirre A, James JH, Keane JM, Wesdorp RIC, Yoshimura N & Westman T: The role of plasma amino acids in hepatic failure. Surgery 38: 276, 1975.

Fischer JE, Rosen HM, Ebeid AM, James JH, Keane JM & Soeters PB: The effect of normalization of plasma amino acids on hepatic encephalopathy in man. Surgery 80: 77, 1976.

Fischer JE: What can we expect from the artificial liver? Intern J Artif Organs 1(4): 187-195, 1978.

Fischer JE: Portasystemic encephalopathy. In: Wright R, Alberti KGMM, Karran S & Millward-Sadlev GH (eds) Liver and biliary disease, pathophysiology, diagnosis, management. Saunders Co, London: Philadelphia: Toronto, Chapter 42, p 973-1001, 1979.

Fisher JE: Amino acids in hepatic coma. Dig Dis Sci, 27: 97-102, 1982.

Flock EV, Block MA, Mann FC, Grindley JH & Bollmann JL: The effect of glucose on the amino acids of plasma after total hepatectomy. J Biol Chem 198: 427-437, 1952.

Flock EV, Block MA, Grindlay JH, Mann FC & Bollman JL: Changes in free amino acids of brain and muscle after total hepatectomy. J Biol Chem, 200: 529-536, 1953.

Flock EV & Bollman JL: Free amino acids in plasma, brain and muscle following hepatectomy. In: Joseph TH (ed) Amino acid pools: distribution, formation and function of free amino acids, Elsevier Publishing Co, 1962.

Flock EV, Tyce GM & Owen CA: Utilization of (U-^{14}C) glucose in brain after total hepatectomy in the rat. J Neuro Chem, 13: 1389-1406, 1966.

Forbes JC & Outhouse EL: Studies on the mechanism of the protective action of xanthine against carbon tetrachloride poisoning. J Pharmacol Exp Ther, 68: 185-193, 1940.

Foster D, Ahmed K & Zieve L: Action of methanethiol on Na^+K^+-ATPase: Implications for hepatic coma. Ann NY Acad Sci, 242: 573, 1974.

Frerichs FT, Offenes Schreiben and den Herrn, Hofrath Dr. Oppolzer in Wien, Wien Med Wochenschr 4: 465, 1854, Cited from Conn HO & Lierthal MM: The hepatic coma syndromes and lactulose. Williams & Wilkins Co, Baltimore, 1979.

Frerichs FT: A clinical treatise on diseases of the liver. (German), 1860, Translated by Murchison C, cited from Frederick S & Bernard FC (eds) Hepatic encephalopathy, Springfield, Ill, Thomas, 1971.

Freund H, Yoshimura N & Fischer JE: Long term therapy with a branched chain amino acid enriched elemental diet. JAMA, 242: 347, 1979.

Fryden A, Weiland O, M'artensson J: Successful treatment of hepatic coma probably caused by acute infectious hepatitis with balanced solutions of amino acids. Scand J Infect Dis, 14(3): 177-180, 1982.

Fürst P, Bergstrom J, Gordon A, Johnsson E & Zimmerman L: Separation of peptides of "middle" molecular weight from biological fluids of patients with uremia. Kidnay Int 7: S272, 1975.

Fürst P, Zimmerman L & Bergstrom J: Determination of endogenous middle molecules in normal and uremic body fluids. Clin Nephrol, 5: 178, 1976.

Gamse R, Mroz E, Leeman S & Lembeck F: The intestine as source of immunoreactive substance P in plasma of the cat. Naunyn-Schmiedeberg's Arch Pharmacol 305: 17-21, 1978.

Garrison FH: History of medicine, 4th ed, Philadelphia, Saunders, p 63, 1961a.

Garrison FH: History of medicine, 4th ed, Philadelphia, Saunders, p 74, 1961b.

Gazzard B.G., Weston M.J., Murray-Lyon I.M., Flax H., Record C.O., Portman B., Langley P.G., Dunlop E.H., Mellon P.J., Ward M.D., William S.R.: Charcoal hemoperfusion in the treatment of fulminant hepatic failure. Lancet, 1,1301, 1974.

Gazzard BG, Portmann B, Murray-Lyon IM & Williams R: Causes of death in fulminant hepatic failure and relationship to quantitative hestological assessment of parenchymal damage. Quart J Med, 44(176): 615-626, 1975a.

Gazzard BG, Hughes RD, Mellon PJ, Portmann B & Williams: A dog model of fulminant hepatic failure produced by paracetamol administration. Br J Exp Path, 56: 408, 1975b.

Geiger A & Yamasaki S: Cytidine and uridine requirements of the brain. J Neuro Chem, 1: 93, 1956.

Geigy Scientific Tables: Diem K & Lentner C (eds), Ciba-Geigy Ltd, Basle, Switzerland, p 554, 1973.

Gelfand MC, Knepshield JH, Cohan S, Ramirez B & Schreiner GE: Treatment of hepatic coma with hemoperfusion through polyacrylamide hydrogen coated charcoal. Kidney Intern, 10: S239, 1976.

Gelfand MC, Winchester JF, Knepshield JH, Cohan SL & Schreiner GE: Biochemical correlates of reversal of hepatic coma with coated charcoal hemoperfusion. Trans Am Soc Artif Intern Organs, XXIV: 239, 1978.

Gerhard H: A quantitative model of cellular regeneration in rat liver after partial hepatectomy. In: Lesch R & Reutter W (eds) Liver regeneration after experimental injury, Stratton Intern Med Book Corp, New York, p 340, 1973.

Gessa GL & Tagliamonte A: Serum free tryptophan: control of brain concentrations of tryptophan and of synthesis of 5-hydroxytryptamina. In: Aromatic amino acids in the brain. Ciba Foundation Symposium 22, p 207-216, 1974.

Gibson TP: Pharmacokinetic determinants of the efficacy of hemoperfusion. In Sideman S & Chang TMS (eds) Hemoperfusion: kidney and liver support and detoxification. Hemisphere Publishing Corp, Washington, p 207, 1980.

Gimson AE, Braude S, Mellon PJ, Canalese J, Williams R. Earlier charcoal hemoperfusion in fulminant hepatic failure. Lancet, Sept: 681-683, 1982.

Giordano C (ed): Sorbents and their clinical applications. Academic Press, New York, 1980.

Glissoni F: Anatomica Hepatis, Amsterdam, p 186, 1659, Cited from Brown H: Hepatic failure, Springfield, Ill, Thomas, p 7, 1970.

Goldberg M, Strocker W, Feeny D & Ruhenstroth-Bauer: Evidence for characterization of a liver cell proliferation factor from blood plasma of partially hepatectomized rats. Horm Metab Res, 12: 94-96, 1980.

Goubeaud G, Leber HW, Schott HH & Schütterle G: Middle molecules and hemoblobin synthesis. Proc Eur Dial Transplant Assoc 13: 371, 1976.

Goulding R: Use of activated absorbent hemoperfusion in acute intoxication. In Chang TMS (ed) Artificial kidney, artificial liver and artificial cells. Plenum Press, p 135-142, 1978.

Granger DN & Perry MA: Permeability characteristics of the microcirculation. In: Mortillaro (ed) The physiology and pharmacology of the microcirculation. Academic Press, 1983.

Gröflin UB & Thölen H: Cerebral edema in the rat with galactosamine induced severe hepatitis. Experientia, 34: 1501, 1978.

Gutman RA, Huang AT & Bouknight NS: Inhibitor of marrow thymidine incorporation from sera of patients with uremia. Kidney Int, 18: 715-724, 1980.

Haag A & Longer K: Reversed phase high-performance liquid chromatography of PTH amino acids. Chromatographia, 7: 659-662, 1974.

Hagstam KE, Larsson LE & Thysell H: Experimental studies on charcoal hemoperfusion in phenobarbital intoxication and uremia. Acta Med Scand, 180: 593, 1966

Hahn M, Masn O, Nencki M & Pawlon J: Die Eck'she fistel zwischen der unteren hohlvene und der pfortader und ihre folgen für den organisonus. Arch Exp Path Pharmakol, 32: 161-210, 1893, Cited from Brown H: Hepatic failure, Charles C Thomas, Springfield, p 9, 1970.

Hamberger A: Amino acid uptake in neuronal and glial cell fractions from rabbit cerebral cortex. Brain Res, 31: 169-178, 1971.

Hannid MA, Mackenzie RL, Jenner RE, Chase RA, Mellon PJ, Trewby PN, Janota I, Davis M, Silk DBA & Williams R: Intracranial pressure in pigs with surgically induced acute liver failure. Gastroenterology 76(1): 123-131, 1979.

Harper HA, Gardner RE, Johansen R, Galante M & McCorkle HJ: Amino acid tolerance in experimental portacaval anastomosis. Surgery, 29: 210-216, 1951.

Hawkins RA, Mans AM & Biebuyck JF: Alterations in amino acid transport across blood brain barrier in rats following portacaval shunting. In Blackburn GL, Grant JP & Young VR (eds) Amino acids, metabolism and medical applications, John Wright PSG Inc, Boston, p 239-253, 1983.

Harrison GG & Smith JS: Massive lethal hepatic necrosis in rats anesthetized with fluroxene, after microsomal enzyme induction. Anesthesiology 39(6): 619-625, 1973.

Heinz E: Transport of amino acids by animal cells. In Hokin LE (ed) Metabolic transport, Academic Press, New York, London, p 455-501, 1972.

ill D.W., Walters F.H., Wilson T.D., Stuart J.D. High performance liquid chromatographic determination of amino acids in the pico-mole range. Analytical Chemistry, 51(8), 1338-1341, 1979.

Holmin T., Agardh C.-D., Alinder G., Herlin P., Hultberg B. The influence of total hepatectomy on cerebral energy state, ammonia-related amino acids of the brain and plasma amino acids in the rat. Europ. J. Clin. Invest., 13,215-220, 1983.

Horowitz ME, Schager DF, Molnar P, Jones EA, Blasberg RG, Patlak CS, Waggoner J & Fenstermacher JD: Increased blood-brain transfer in a rabbit model of acute liver failure. Gastroenterology 84(5): 1003-1011, 1983.

Hörtnagl H, Singer EA, Lenz K, Kleinberger G & Lochs H: Substance P is markedly increased in plasma of patients with hepatic coma. Lancet, p 480-483, 1984.

Hoyumpa AM Jr, Desmond PV, Avant GR, Roberts RK & Schenker S: Hepatic encephalopathy. Gastroenterology, 76: 184-195, 1979.

Hughes R, Ton HY, Langley P, Davies M, Hanid MA, Mellon P & Silk DBA: Albumin coated amberlite XAD-7 resin for hemoperfusion in acute liver-failure: 2, in vivo evaluation. Artificial organs, 3: 23-26, 1979.

Iob V, Coon WW & Sloan M: Altered clearance of free amino acids from plasma of patients with cirrhosis of the liver. J Surg Res, 6: 233-239, 1966.

Iversen LL: Substance P. British Medical Bulletin, 38(3): 277-282, 1982.

James JH, Hodgman JM & Fischer JE: Alterations in brain octopamine and brain tyrosine following portacaval anastomosis in rats. J Neurochem, 27: 223, 1976.

James JH, Jeppson B, Ziparo V & Fischer JE: Hyperammonaemia, plasma amino acid imbalance, and blood brain amino acid transport: a unified theory of portal systemic encephalopathy. Lancet, 2: 772, 1979.

James JH & Fischer J: Transport of neutral amino acids at the blood brain barrier. Pharmacology, 22: 1-7, 1981.

Jones EA: The enigma of hepatic encephalopathy. Postgraduate Medical J, 59(54): 42-54, 1983.

Jones EA, Schafer DF, Ferenci P & Pappas SC: The neurobiology of hepatic encephalopathy. Hepatology, 4(6): 1235-1242, 1984.

Johnston DG, Alberti KGMM: The liver and the endocrine system. In: Wright R, Alberti KGMM, Karrans, Millward-Sadler GH (eds) Liver and biliary diseases, Saunders Co Ltd, London: Philadelphia: Toronto, p 134, 1979.

Kawanishi H, Nishiki M, Sugiyama M, Kimura S, Tsuchiya T & Ezaki H: Analysis of hepatic failure plasma by high performance gel-chromatography. Hiroshima J Med Sci, 32(4): 451-454, 1983.

Keppler D, Lesch R, Renter W & Decker K: Experimental hepatitis induced by D-galactosamine. Exp Mol Pathol, 9: 279, 1968.

Keppler D & Decker K: Studies on the mechanism of galactosamine hepatitis: accumulation of galactosamine-1-phosphate and its inhibition of UDP-glucose pyrophosphorylase. Eurpean J Biochem, 10: 219-225, 1969.

Klasing KC & Austic RE: Changes in plasma, tissue, and urinary nitrogen metabolites due to an inflammatory challenge. Proc Soc Exp Biol Med, 176: 276-284, 1984.

Kline DG, Doberneck RC, Chun BK & Rutherford RB: Encephalopathy in graded portacaval shunts. Ann Surg 164: 1003, 1966.

Kranz JC, Carr CJ, Lu G & Bell FK: The anaesthetic action of trifluorethyvinylether. J Pharmacol Exp Ther, 108: 488-495, 1953.

Krnjevic K & Phillis JW: Iontophoretic studies of neurons in the mammalian cerebral cortex. J Physiol, 165: 274-304, 1963.

Kühne W (1858): Cited from Brown IA: Liver-brain relationships, Springfield, Thomas, p 69, 1957.

Kuster GGR & Woods JE: Auxiliary liver transplantation in the dog as temporary support in acute fulminant hepatic necrosis. Annals Surg, 176(6): 732-735, 1972.

Lajtha: Cerebral passage of free amino acids. In Holden JT (ed) Amino acid pools. Elsevier Publishing Co, Amsterdam, p 554-563, 1962.

Lajtha A, Blasberg R & Levi G: Control of cerebral amino acid concentrations. In Leathem JH (ed) Protein nutrition and free amino acid patterns, Rutgers University Press, New Brunswick, p 187, 1965.

Lajtha A: Amino acid transport in the brain, in vivo and in vitro. In: Aromatic amino acids in the brain, Ciba Foundation Symposium 22, Elsevier, Amsterdam, p 25-49, 1973

Lal S, Aronoff A, Garelis E, Sourkes TL, Young SN & dela Vega CE: Cerebral fluid homovanillic acid, 5-hydroxyindoleacetic acid, lactic acid, and pH before and after probenecid in hepatic coma. Clin Neurol Neurosurg, 2: 142-154, 1974

Lamson PD & Wing R: The effect of carbon tetrachloride and alcohol on the acid base balance of the blood. J Biological Chem, 69: 349-355, 1926.

Landis EM & Pappenheimer JR: Exchange of substances through the capillary walls. In: Hamilton WF & Dow P (eds) Handbook of Physiology, Section 2: Circulaton. Vol II, p 961-1034, Amer Physiol Soc, Washington DC, 1963.

Laursen H: Cerebral vessels and glial cells in liver disease, a morphometric and electron microscopic investigation. Acta Neurol Scandinav, 65: 381-412, 1982.

Laursen H & Westergaard E: Enhanced permeability to horseradish peroxidase across cerebral vessels in the rat after portocaval anastomosis. Neuropath Appl Neurobiol, 3: 29-43, 1977.

Leber HW, Goubeaud G & Spiegelhalter G: Middle molecules in renal failure. Ann Clin Biochem, 19: 389, 1979.

Leber HW, Klansman J, Goubeaud G & Schutterle G: Middle molecules in the serum of patients and rats with liver failure: influence of sorbent hemoperfusion. In: Brunner G & Schmidt FW (eds) Artificial Liver Support. Berlin: Springer-Verlag, p 96-103, 1981.

Lee SH & Fisher B: Portacaval shunt in the rat. Surgery 50: 668, 1961.

Lee JC & Bakay L: Electron microscopic studies on experimental brain edema. In: Klatzo I & Seitelberger F (eds) Brain Edema. Berlin: Springer-Verlag, 590-597, 1967.

Lembeck F, Holzer P, Schwedifsch M & Gamse R: Elimination of substance P from the circulation of the rat and its inhibiton by bacitracin. Naunyn Schmiedebergs Arch Pharmacol, 305: 9-16, 1978.

Lesch R, Reutter W. Keppler D & Decker K: Liver restitution after acute galactosamine hepatitis: autoradiographic and biochemical studies in rats. Exp Molec Path, 12: 58-69, 1970.

Levin V & Patlak CS: A compartmental analysis of ^{24}Na kinetics in rat cerebrum, sciatic nerve and cerebrospinal fluid. J Physiol (London) 224: 559-581, 1972.

Liehr H, Grün M, Seelig H-P, Seelig R, Reutter S & Heine W-D: On the pathogenesis of galactosamine hepatitis: indications of extracellular mechanisms responsible for liver cell death. Virchows Archiv B Cell Path, 26: 331-344, 1978.

Livingstone AS, Potvin M, Goresky CA, Finlayson MH & Hinchey EJ: Changes in the blood brain barrier in hepatic coma after hepatectomy in the rat. Gastroenterology, 73: 697-704, 1977.

Loda M, Clowes GHA, Nespoli A, Bigatellol, Birkett DH & Menzoian JO: Encephalopathy, oxygen consumption, visceral amino acid clearance and mortality in cirrhotic surgical patients. Am J Surgery, 147: 542-550, 1984.

Lorenzo AV: Amino acid transport mechanisms of the cerebrospinal fluid. Federation Proc, 33: 2079-2085, 1974.

Lorenzo AV, Somoly-Caruthers J & Greene E: Development of amino acid transport mechanisms in the choroid plexus. In: Cserr HF, Macher F & Fend V (eds) Fluid environment of the brain. Academic Press Inc, New York, 1975.

Löscher W: GABA in plasma, CSF and brain of dogs during acute and chronic treatment with gamma-acetylenic GABA and valproic acid. In: Okada Y & Roberts E (eds) Problems in GABA research from brain to bacteria. Excerpta Medica, Amsterdam, p 102, 1982.

Lutz W, Markiewicz K & Klyszejko-Stefanowicz L: Oligopeptides in blood plasma of healthy humans and of patients with nephrotic syndrome. Clinica Chimica Acta, 39: 319-326, 1972a.

Lutz W, Markiewicz K & Klyszejko-Stefanowicz L: Oligopeptides excreted in the urine of healthy humans and of patients with nephrotic symdrome. Clinica Chimica Acta, 39: 425-431, 1972b.

Madden JW, Gertman PM & Peacock EE Jr: Dimethylnitrosamine-induced hepatic necrosis: a new canine model of an ancient disease. Surgery 68: 260, 1970.

Mariolo AT, Bianchi-Porro G, Galli C, Sessa M & Polli EE: Brain energy metabolism is hepatic coma. In: Polli EE (ed) Neurochemistry of hepatic coma. Karger Basel, 1971.

Makowka L, Falk RE, Rotstein LE, Falk JA, Nossal N, Langer B, Blendis LM & Phillips MJ: Reversal of experimental acute hepatic failure in the rat. J Surg Res, 29: 479-487, 1980.

Malchesky PS, Varnes W, Nokoff R & Nose Y: The charcoal capllary hemoperfusion system. Proc Eur Dial Transplant Assoc 13: 242-249, 1976.

Man NK, Terlain B, Paris J, Werner G, Sausse A & Funck-Brentano JL: An approach to "middle molecules" identification in artificial kidney dialysate, with reference to neuropathy prevention. Trans Am Soc Artif Int Organs, 19: 320, 1973.

Mann FC: Studies in the physiology of the liver, I. technique and general effects of removal. Am J M Soc, 161: 37, 1921.

Mann FC: Effect of complete and partial removal of the liver. Medicine, 6: 419, 1927.

Mann FC & Magath TS: Studies on the physiology of the liver, II. The effect of the removal of the liver on the blood sugar level. Arch Int Med, 30: 73, 1922.

Mans AM, Biebuyck JF, Saunders SJ, Kirsch RE & Hawkins RA: Tryptophan transport across the blood brain barrier during acute hepatic failure. J Neurochem, 33: 409-418, 1979.

Marchesini G, Zoli M & Dondi C: Anticatabolic effect of branched-chain amino acid enriched solutions in patients with liver cirrhosis. Hepatology, 2(4): 420-425, 1982.

Marsden CA & Curzon G: The contribution of tryptamine to the brhavioural effects of L-tryptophan tranylcypromine-treated rats. Psychopharmacology, 57: 71-76, 1978.

Mason EC & Davidson CS: A study of tissue autolysis in vivo: I. Blood changes physical and chemical. J Lab Clin Med, 10: 622, 1924-5.

Mattson W Jr, Iob V, Slaan M, et al: Alterations of individual free amino acids in brain during acute hepatic coma. S G O, 130: 263, 1970.

McClain CJ, Zieve L, Doizaki W, Gilberstadt S & Onstad G: Mercaptans in portal systemic encephalopathy (PSE) due to alcoholic liver disease. Gastroenterology, 74: 1064, 1978.

McDermott WV Jr & Adama RD: Episodic stupor associated with an Eck fistula in the human with particular reference to the metabolism of ammonia. J Clin Invest, 33: 1-9, 1954.

McLean AEM: The measurement of liver injury and protection, with special reference to paracetamol, dimethylnitrosamine and carbon tetrachloride. In: Keppler D (ed) Pathogenesis and mechanisms of liver cell necrosis, University Park Press, Baltimore, 1974.

McMenamy RH, Vang J & Drapanas T: Amino acid and L-keto acid concentration in plasma and blood of the liverless dog. Amer J Physiol, 209: 1046-1052, 1965.

McMenamy RH, Shoemaker WC< Richard JE & Elwyn D: Uptake and metabolism of amino acids by the dog liver perfused in situ. Am J Physiol 202(3): 407-414, 1962.

Meak JL & Neff HH: Is cerebrospinal fluid the major avenue for the removal of 5-hydroxyindoleacetic acid from the brain? Neuropharmacology, 12: 497-499, 1973.

Medline A, Schaffner F & Popper H: Ultrastructural features in galactosamine-induced hepatitis. Exp Molec Path, 12: 201-211, 1970.

Michel H, Solere M, Granier P, Lauret G, Bali JP, Pons F & Bellet-Herman H: Treatment of cirrhotic hepatic encephalopathy with L-DOPA, a controlled trial. Gastroenterology, 79: 207, 1980.

Migone L, Dall'aglio P & Buzio C: Middle molecules in uremic serum, urine and dialysis fluid. Clin Nephrol, 3: 82-93, 1975.

Miller LL: The role of the liver and the nonhepatic tissue in the regulation of free amino acid levels in the blood. In: Holden JT (ed): Amino acid pools. Amsterdam:Elsevier p 708-721, 1962.

Misra MK, P'eng F-K, Sayhoun A, Kashii A, Perry CD, Caridis T & Slapak M: Acute hepatic coma: a canine model. Surgery, 72: 634-642, 1972.

Mitchell JR, Potter WZ & Jollow DJ: Furosemide-induced hepatic and renal tubular necrosis: I. effects of treatments which alter drug metabolizing enzymes. Fed Proc, 32: 305, 1973.

Monier D & Wagle SR: Studies on gluconeogenesis in galactosamine induced hepatitis. Proc Soc Exp Biol Med, 136: 377-380, 1971.

Munro HN: Control of plasma amino acid concentrations. In: Aromatic amino acids in the brain, Ciba Foundation Symposium 22, Elsevier Excerpta Medica, North-Holland, p 5-24, 1974.

Morgan MY, Milsom JP & Sherlock S: Plasma ratio of valine, leucine and isoleucine to phenylalanine and tyrosine in liver disease. Gut, 19: 1068-1073, 1978.

Muirhead EE & Reid AF: A resin artificial kidney. J Lab Clin Med, 33: 841-844, 1948.

Newsholme EA & Leech AR: Biochemistry for the medical sciences. John Wiley & Sons, p 398-399, 1983.

Nishiki M, Sagiyama M, Tsuchiya T & Ezaki H: Influence of hemoperfusion using coated bead-type charcoal on middle molecules of hepatic failure dogs. Hiroshima J Medicine 33(3): 493-497, 1984.

Niu Z, Jia SR, Zhang DY, Xu CX, Tang XJ, Fan WK, Luo YP, Li ZM: The effects of hemoperfusion in galactosamine induced FHF rats. Chungking Med College Bull, Nov: 1-6, 1980.

Norenberg MD: A light and electron microscopic study of experimental portalsystemic (ammonia) encephalopathy. Lab Invest, 36: 618-627, 1977.

Odaka M, Tabata Y, Kobayashi H, Nomura Y, Soma H, Hirasawa H & Sato H: Clinical experience of bead-shaped charcoal hemoperfusion in chronic renal failure and fulminant hepatic failure. In: Chang TMS (ed) Artificial Kidney, Artificial Liver, and Artificial Cells, New York: Plenum Press, p 79-88, 1978.

Oettel HJ: Folgen der leberinsnffizienz. Schweiz Med Wschr, 78: 833, 1948.

Ogihara K, Mozai T & Hirai S: Tryptophan as cause of hepatic coma. New Engl J Med, 275: 1255-1256, 1966.

Oldendorf WH & Davson H: Brain extracellular space and the sink action of cerebrospinal fluid. Arch Neurol, 17: 214-218, 1967.

Oldendorf WH & Szabo J: Amino acid assignment to one of three blood brain barrier amino acid carriers. Am J Physiol, 230(1): 94, 1976.

Oldendorf WH: Stereospecificity of blood brain barrier permeability to amino acids. Am J Physiol, 224: 967, 1973.

Olsson AG, Carlsson LA, Jogestrand T & Kaijser L: The effect of prostaglanding E intraarterially in peripheral artery disease. In: Louhija A, Valtoner V (eds) Internal medicine: 1976 topics, Karger, Basel, p 237-241, 1977.

O'neill PL, Blanc PL & Sutherland DER: Factors effective in reducing rat mortality due to acute liver failure as induced by D-galactosamine poisoning. J Surg Res, 36: 372-376, 1984.

Opolon P, Huguet CI, Nasinovici V, Lavallard MC, Crubille c, Gallot D, Bloch P &Granger A: Effect of middle molecule removal on encephalopathy during acute liver failure. Trans European Society Artif Intern Organs, 2: 228, 1975.

Opolon P, Rapin JR, Huguet S, Granger A, Delorme ML, Boschat M & Sausse A: Hepatic failure coma (HFC) treated by polyacrylonitrile membrane (PAN) hemodialysis (HD). Trans Am Soc Artif Intern Organs, 22: 701-710, 1976a.

Opolon P, Lavallard MC, Huguet CL, Bidallier M, Granger A, Gallot P & Bloch P: Hemodialysis versus cross hemodialusis in experimental hepatic coma. Surg Gynecol Obstet, 142: 845, 1976b.

Opolon P: Large-pore hemodialysis in fulminant hepatic failure. In: Brunner G & Schmidt FW (eds) Artificial liver support, Springer-Verlag: Berlin: Heidelberg: New York, p 141, 1981.

Ouchi K, Okabe K, Asanuma Y, Koyama K & Sato T: Effects of homologous plasma cross-circulation on liver function in galsctosamine-induced hepatic necrosis in rats. Artificial Organs 8(2): 179-185, 1984.

Pappas SC, French P, Schaffer DF & Jones AE: Visual evoked potentials in a rabbit model of hepatic encephalopathy: hyperammonemia, postictal, synergistic neurotoxins. Gastroenterology, 86: 546-551, 1984.

Paul SM, Marangos PJ & Skolnick P: The benzodiazepin-GABA-chloride ionophore receptor complex: common site of minor tranquilliser action. Biol Psychiatry, 16: 213-229, 1981.

Petit JM & Biggs JT: Tricyclic antidepressant overdoses in adolescent patients. Pediatrics, 59: 283-287, 1977.

Pickering RW, James GWL & Parker FL: An investigation of some parameters that affect the galactosamine model of hepatitis in the rat. Arzreim-Forsch (Drug Res), 25(6): 898-901, 1975.

Plum F & Hindfelt B: The neurological complications of liver disease. In: Vinken BJ & Bruyn GW in collaboration with Klawans HL (eds) Handbook of clinical neurology, 27, Metabolic and deficiency diseases in the nruvous system, New York, American Elsevier Publishing, p 349-377, 1976.

Potter WZ, Nelson WL, Thorgeirsson SS, Sasame H, Jollow DJ & Mitchell JR: Furosemide-induced hepatic necrosis, II. Comparison of necrosis with covalent binding of furosemide. Fed Proc 32: 305, 1973.

Potvin M, Finlayson M.H., Hinchey E.J., Lough J.O., Goresky C.A. Cerebral abnormalities in hepatectomized rats with acute hepatic coma. Lab Invest. 50:560-564, 1984.

Prescott LF, Wright N, Roscol P & Brown SS: Plasma paracetamol half life and hepatic necrosis in patients with paracetamol overdosage. Lancet, 1: 519-522, 1971.

Purpura DP: Intracellular studies of synaptic organizations in the mammalian brain. In: Pappas GD & Purpura DP (eds) Structure and function of synapses. New York: Reven, p 257-302, 1972.

Quarfoth G, Ahmed K, Foster D & Zieve L: Action of methanethiol in membrane (Na^+, K^+)-ATPase of rat brain. Biochem Pharmacol, 25: 1039-1044, 1976.

Quastel JH: Enzymic activity of the brain. Proc R Soc Med, 29(1): 200, 1935.

Rake MO, Flute PT, Pannell G & Williams R: Intravascular coagulation in acute hepatic necrosis. Lancet, 1: 533-537, 1970.

Rappaport AM: Experimental ischemia of the liver and hepatic coma. Transactions of 10th liver injury conference, New York: Josiah Macy Jr Foundation, p 146-179, 1951.

Rappaport AM, McDonald MH & Boronny ZJ: Hepatic coma following ischaemia of the liver. SGO, 97: 748, 1953.

Rapoport SI: Blood Brain Barrier in Physiology and Medicine. Raven Press. New York, 1976.

Record CO, Buxton B, Chase RA, Curzon G, Murray-Lyon IM & Williams R: Plasma and brain amino acids in fulminant hepatic failure and their relationship to hepatic encephalopathy. Eur Clin, 6: 387, 1976.

Record CO, Al Mardini H & Bartlett K: Blood and brain mercaptan concentrations in hepatic encephalopathy. Hepatology 2: 145, 1982.

Reinhardt WO & Bazell AH: One stage functional hepatectomy in the rat. Proc Soc Exp Biol Med, 62: 270, 1946.

Reutter W, Bauer C, Bachmann W & Lesch R: The galactosamine refractory regenerating rat liver. In: Lesch R & Reutter W (eds) Liver regeneration after experimental injury, Stratton Intern Med Book Corporation, New York, 1973.

Rigotti P, Zanchin G, Vassanelli P, Bettineschi F, Dussini N & Battistin L: Cerebral amino acid levels and transport after portacaval shunt in the rat: effects of liver arterialization. J Surg Res, 33: 415-422, 1982.

Ritt DJ, Whelan G, Werner DJ, Eigenbrodt EH, Schenker S & Combes B: Acute hepatic necrosis with stupor or coma. Medicine, 48: 151, 1969.

Roberts E & Simonsen DG: Free amino acids in animal tissue. In: Holden JT (ed) Amino acid pools, Amsterdam: Elsvier, p 284-349, 1962.

Rogers QR & Harper AE: Significance of tissue pools in the interpretation of changes in plasma amino acid concentrations. In: Leathem JH (ed) Protein nutrition and free amino acid patterns, Rutgers University Press: New Brunswick, p 107, 1965.

Rokitansky C: A manual of pathological anatomy. Vol II. the abdominal viscera, 1855, Cited from Conn HO & Lieberthal MM: The hepatic coma syndrome and lactulose. Williams & Wilkins Co. Baltimore, p 95, 1979.

Rosen HM, Yoshimura N, Hodgman JM & Fischer JE: Plasma amino acid patterns in hepatic encephalopathy of differing etiology. Gastroenterology, 72: 483-487, 1977.

Rosenbaum JL: Poisonings. In: Giordano C (ed) Sorbents and their clinical aplications, Academic Press, New York, p 451-467, 1980.

Rossi-Fanelli F, Freund H, Krause R, Smith AR, James H, Gastorina-Ziparo S & Fischer JE: Induction of coma in normal dogs by the infusion of aromatic amino acids and its prevention by the addition of branched chain amino acids. Gastroenterology, 83: 664-671, 1982.

Rossier JP & Bloom FE: Distribution of opioid peptides. In: Malick JB & Bell RMS (eds) Endorphins. Marcel Dekker Inc, New York & Basel, p 89-112, 1982.

Roth M: Fluorescence reaction for amino acids. Analyt Chem, 43: 880-882, 1971.

Rubin E, Kohan P, Tomita F & Jacobson JH: II. Experimental hepatic siderosis following portacaval shunt. Proc Soc Exp bol Med, 115: 350, 1963.

Rueff B & Benhamou J-P: Progress report: Acute hepatic necrosis and fulminant hepatic failure. Gut, 14: 805-815, 1973.

Ryan CJ, Courtney JM, Klinkman H & Blumgart LH: Partial hepatectomy and subsequent charcoal hemoperfusion in the conscious rat. In: Brunner & Schmidt (eds) Artificial liver support, Apringer-Verlag, Berlin: Heidelberg: New York, 1981.

Saunders SJ, Seggie J, Kirsch RE & Terblanche J: Acute liver failure. In: Wright R, Alberti KGMW, Karran S & Willward-Sedler GH (eds) Liver and biliary diseases, Chapter 27, Saunder WB Co Ltd, London: Philadelphia: Toronto, 1979.

Schaefer DF & Jones EA: Hepatic encephalopathy and the gamma-amino butyric acid neurotransmitters system. Lancet, 1: 18, 1982.

Schafer DF, Pappas SC, Brody LE, Jacobs R & Jones EA: Visual evoked potentials in a rabbit model of hepatic encephgalopathy, I. Sequential changes and comparisons with drug-induced comas. Gastroenterology, 86: 540-545, 1984.

Schenker S, McCandless DW & Brophy E: Studies on intracerebral toxicity of ammonia. J Clin Invest, 46: 838-848, 1967.

Schindhelm K & Farrell PC: Patient-hemodialyzer interactions. Trans Am Soc Artif Intern Organs, 24: 357-366, 1978.

Seda HWM, Hughes RD, Gove CD & Williams R: Removal of inhibitors of brain Na^+,K^+-ATPase by hemoperfusion in fulminant hepatic failure. Artificial Organs, 8(2): 174-178, 1984.

Serrou B, Coburg AJ, Abouna GM, & Aldrete JA: Hemodynamic and metabolic stability after total hepatectomy in the dog. Int Surgery, 55: 235-242, 1971.

Seta K, Sansurm & Lajtha A: The rate of incorporation of amino acids into brain proteins during infusion in the rat. Biochim Biophys Acta, 294: 472-480, 1973.

Sherlock S: Neuropsychiatric changes following porta-systemic shunting. Colston papers: The liver, Butterworth Scientific Publications, 241, 1967.

Sherlock S: Diseases of the liver and biliary system. Chapter 6, Hepatic pre-coma and coma, Blackwell Scientific Publications, 1975.

Shi ZQ & Chang TMS: The effects of hemoperfusion using coated charcoal or tyrosinase artificial cells on middle molecules and tyrosine in brain and serum of hepatic coma rats. Trans Am Soc Artif Intern Organs, 28: 205, 1982.

Shi ZQ & Chang TMS: In vitro adsorption spectrum of plasma amino acids by coated charcoal hemoperfusion. Int J Artif Organs, 6(5): 267-270, 1983

Shi ZQ & Chang TMS: Amino acid disturbances in experimental hepatic coma rats. Int J Artif Organs, 7(4): 197-202, 1984a.

Shi ZQ & Chang TMS: Effect of charcoal hemoperfusion on amino acid disturbance in experimental hepatic coma. International Journal of Artificial Organs 7(4): 203-208, 1984b.

Shi ZQ & Chang TMS: Amino acid distubances in hepatic coma rats and the effects of charcoal hemoperfusion. Proceedings of 5th International Symposium on Hemoperfusion and Artificial Organs, Tianjin, People's Republic of China, 1983.

Shu CD & Chang TMS: Tyrosinase immobilized within artificial cells for detoxification in liver failure: I. preparation and in vitro studies. Int J Artif Organs, 3: 287, 1980.

Shu CD & Chang TMS: Tyrosinase immobilized within artificial cells for detoxification in liver failure, II. in vivo studies in fulminant hepatic failure rats. Int J Artif Organs, 4: 81, 1981.

Shu CD, Chang TMS: Middle molecules in the serum, brain and urine of galactosamine-induced hepatic failure rats. Int J Artif Organs, 6(5): 273-277, 1983.

Shultz SG & Curran PF: Coupled transport of sodium and organic solutes. Physiol Review, 50(4): 637-718, 1970.

Sideman S & Chang TMS (eds): Hemoperfusion: kidney and liver support and detoxification. Hemisphere, Washington, 1980.

Silk DBA, Hanid MA, Trewby PN, Davies M, Chase RA, Langlay PG, Mellon PJ, Wheeler PG & Williams R: Treatment of fulminant hepatic failure by polyacrylonitrile-membrane hemodialysis. Lancet, 2: 1-3, 1977.

Silk DBA, Williams R: Experience in the treatment of fulminant hepatic failure by conservative therapy charcoal hemoperfusion, and polyacrylonitrile hemodialysis. Int J Artif Organs, 1: 29, 1978.

Silk DBA, Williams R: The liver: acute liver failure. Brit J Hospital Med, Nov, p 437, 1979.

Simons SS Jr, & Johnson DF: The structure of the fluorescent adduct formed in the reaction of o-phthaldialdehyde and thiols with amines. J Am Chem Soc, 98: 7098-7099, 1976.

Singer C: The evolution of anatomy. Kegan, Paul, Trench, Trulner and Co Ltd, p 58-61, 1925.

Skrabanek P, Cannon D, Kirrane J, Legge D & Powell D: Circulating immunoreactive substance P in man. Ir J Med Sci 145: 399-408, 1976.

Skrabanek P & Powell D: Substance P. Vol 2, Chapter 4, Eden Press, p 27, 1980.

Smialowski A: The effect of intra-hippocampal administration of gamma-amino butyric acid (GABA). In: Fonnum F (ed) Amino acids as chemical transmitters, New York: Plenum Press, 1977-1980, 1978.

Smith A., Rassi-Fanelli F.: Alterations in plasma and CSF amino acids, amines and metabolites in hepatic coma. Ann. Surg., 187,343-350, 1978.

Sourkes TL: Tryptophan in hepatic coma. J Neural Transmission Suppl 14: 79-85, 1978.

Soeters PB & Fischer JE: Insulin, glucagon, amino acid imbalance and hepatic encephalopathy. Lancet, 2: 88-882,1976.

Stadie WC & Van Slyke DD: The effect of acute yellow atrophy on mechanism and on the composition of the liver. Arch Int Med, 25: 693, 1920.

Starzel TE, Bernhard VM, Cortes N & Benevenuto R: A technique for one-stage hepatectomy in dogs. Surgery, 46: 880, 1959.

Stenger RJ & Johnson EA: Effects of phenobarbital pretreatment on the responses of rat liver to halothane administration. Proc Soc EXp Biol Med, 140: 1319-1324, 1972.

Stöker E & Wullstein HK: Capacity of liver regeneration after partial hepatectomy in cirrhotic and CCl_4-intoxicated old rats. In: Lesch R & Reutter W (eds) Liver regeneration after experimental injury, Stratton Intern Med Book Corporation, New York, p 60, 1973.

Swank RT & Munkres KD: Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Analytical Biochem, 39: 462-477, 1971.

Tabata Y & Chang TMS: Comparison of six artificial liver support regimes in fulminant hepatic coma rats. Trans Am Soc Artif Intern Organs, 26: 384, 1980.

Takahashi N, Miyazaki K, Koide O, Kamamura T, Yoshitake S & Sambe K: On the effect of the uracil-D riboside (uridine) on the liver lesions. Japanese G E J Proc, 47th Ann Meet, Part 1, p 87, 1961.

Takahashi Y: Serum lipids in liver disease and the relationship of serum lipids and hepatic coma. Japanese J Gastroenterlogy, 60: 571-579, 1963.

Terblanche J, Hickman R, Miller DJ & Saunders SJ: Animal experience with support systems: are there appropriate animal models of fulminant hepatic necrosis. In: Williams R & Murray-Lyon IM (eds) Artificial liver support, Pitman Medical, Kent, p 163-172, 1974.

Thysell H, Lindholm T, Heinegard D, Jonsson E, Nylen U, Svensson T, Bergvist G & Gullberg CA: A hemoperfusion column using cellophane coated charcoal. Proc Eur Soc ARtif Organs, 2: 63-68, 1975.

Tiselius A: (1944) Cited from Greenstein & Winitz (eds) Chemistry of the amino acids, Vol II. John Wiley & Sons Inc, p 1448, 1961.

Trewby RN, Casemore C & Williams R: Continuous bipolar recording of the EEG in patients with fulminant hepatic failure. Electroenceph Clin Neurophysiol, 45: 107-110, 1978.

Trey C, Burns DG & Saunders SJ: Treatment of hepatic coma by exchange blood transfusions. New Eng J Med, 274: 473-481, 1966.

Trey C, Garcia FG, King NW, Lowenstein LM & Davidson CS: Massive liver necrosis in the monkey: the effects of exchange blood transfusion on fulminant liver failure. J Lab Clin Med, 73: 784-794, 1969.

Trey C & Davidson CS: The management of fulminant hepatic failure. In: Popper H, Schaffner F (eds) Progress in liver diseases. Chapter 18, Grune & Stratton: New York, p 282, 1970.

Tricklebank MD, Bloxam DL & Curzon G: Effects of portocaval anastomosis on behaviour and brain tryptophan metabolism. J Neural Transmission, Suppl 14, p 69-78, 1978.

Tyce GH, Flock EV, Owen CA, Stobie GH, & David C: 5-Hydroxyindole metabolism in the brain after hepatectomy. Biochem Pharmacol, 16: 979-992, 1967.

Van Slyke DD & Meyer GM (1912): Cited from Christensen HN: Mode of transport of amino acids into cells. In: McElroy WD & Glass HB (eds): Amino acid metabolism, Baltimore: Johns Hopkins Press, p 63-106, 1955.

Van Leenhoff JW, Hickman R, Saunders SJ & Terblanche J: Massive liver cell necrosis-induced in the pig with carbon tetrachloride. 48: 1201-1204, 1974.

Van Steirteghem AC & Young DS: Chapter 11. Amino acids in physiological fluids. In: Blackburn S (ed) Amino acid determination, methods and techniques, 2nd Ed, Marcel Dekker Inc, New York, p 281, 1978.

Wahren J, Denis J, Desurmont P, Eriksson LS, Escoffier J-M, Gauthier AP, Hagenfeldt L, Michel H, Opolon P, Paris J-C & Veyrac M: Is intravenous administration of branched chain amino acids effective in the treatment of hepatic encephalopathy? A multicenter study. Hepatology, 3(4): 475-480, 1983.

Walker S, Grotz R, Czygan P, Stiehl A, Lanzinger G, Sieg A, Racdsch R & Kommerell B: Oral keto analogs of branched chain amino acids in hyperammonemia in patients with cirrhosis of the liver: a double blind crossover study. Digestion, 24(2): 105-111, 1982.

Walser M: Urea metabolism regulation and sources of nitrogen. In: Blackburn GL, Grant JP & Young VR (eds) Amino acid metabolism and medical applications. John Wright, PSG Inc, Boston, p 77-87, 1983.

Walshe JM: Observation on symptoms and pathogenesis of hepatic coma. Quart J Med, 20: 421, 1951.

Walshe JM: Disturbances of amino acid metabolism following liver injury: a study by means of paper chromatography. Quart J Med, 22: 483, 1953.

Ware AJ, D'Agostino AN, Combes B: Cerebral edema: a major complication of massive hepatic necrosis. Gastroenterology, 61(6): 877-884, 1971.

Weber FL Jr & Reiser BJ: Relationship of plasma amino acids to nitrogen balance and portal-systemic encephalopathy in alcoholic liver disease. Dig Dis Sci, 27(2): 103-110, 1982.

Weber WJ Jr, Asce AM & Morris JC: Kinetics of adsorption on carbon from solution. J Sanit Engin Div, April: 31-57, 1963.

Weil-Malherbe H: Studies on brain metabolism, 1. the metabolism of glutamic acid in brain. Biochem J, 30: 665, 1936.

Widmann JJ & Fahimi HD: The regenerative response of Kupffer cells and endothelial cells after partial hepatectomy. In: Lesch R & Reutter W (eds) Liver regeneration after experimental injury, Stratton Intern Med Book Corp, New York, p 89, 1973.

Wilcox WH: Lettsomiar lectures on jaundice with special reference to types occurring during the war. Tr M Soc London, 42: 147, 1919.

Williams R & Murray-Lyon IM (eds): Artificial liver support, Pitman Medical, Kent, London, 1975.

Williams R: Hepatic failure and development of artificial liver support system. In: Popper H & Schaffner F (eds) Progress in liver disease, Grune & ktratton: New York, Vol 5, p 418-435, 1976.

Wilmore DW, Brooks DC, Muhlbacher F, Kapadia CR, Aoki TT & Smith R: Altered amino acid concentrations and flux following traumatic injury. In: Blackburn GL, Grant JP & Young VR (eds) Amino acids metabolism and medical applications. John wright PSG Inc, Boston, p 387, 1983.

Winchester JF, Apliga MT & Kennedy AC: Short term evaluation of charcoal hemoperfusion combined with dialysis in uremia patients. Kidney Int, 10: S315, 1976.

Winchester JF, Gelfand MC, Knepshield JH & Schreiner GE: Dialysis and hemoperfusion of poisons and drugs -update. Trans Am J Intern Organs, 23: 762-842, 1977.

Winchester JF, Gelfand MC: The artificial liver 1978. Int J Artif Organs, 2: 3, 1979.

Winchester JF: Hemoperfusion in uremia In: Giordano C (ed) Sorbents and their clinical applications, Academic Press, New York, p 387-413, 1980.

Wood JH: Neurobiology of cerebrospinal fluid. Plenum Press, New York, London, p 45, 1980.

Wustrow TH, Hoorn-Hickman RV, van Hoorn WA, Vinik AL, Fischer M & Terblanche J: Acute hepatic ischemia in the pig - the changes in plasma hormones, amino acids and brain biochemistry. Hepato-gastroenterology, 28: 143-146, 1981.

Yager JD Jr, Hopkins HA, Campbell HA & Potter VR: An autoradiographic analysis of DNA synthesis following partial hepatectomy in rats. In: Lesch & Reutter W (eds) Liver regeneration after experimental injury, Stratton Intern Med Book Corp, New York, p 56, 1973.

Yatzidis H: A convenient hemoperfusion microapparatus over charcoal for the treatment of endogenous and exogenous intoxications - its use as an effective artificial kidney. Proc Eur Dial & Transplant Assoc, 1: 83, 1964.

Young SN, Lal S, Feldmuller F, Sourkes TL, Ford RM, Kiely M & Martin JB: Parrallel variation of ventricular CSF tryptophan and free serum tryptophan in man. J Neurol Neurosurg & Psychiatry, 39: 61-65, 1976.

Young SN & Lal S: CNS tryptamine metabolism in hepatic coma. J Neural Transmission, 47: 153-161, 1980.

Yu YT & Chang TMS: The effects of polymer-solvent compositions on the formation of collodion membrane artificial cells. Biomat Med Dev Art Org, 8(3): 273-281, 1980.

Zaki AEO, Wardle EN, Canalese J, Ede RJ & Williams R: Potential toxins of acute liver failure and their effects on blood-brain barrier permeabiity. Experientia, 39:988-91, Sept, 1983.

Zaki AEO, Ede RJ, Davis M & Williams R: Experimental studies of blood brain barrier permeability in acute hepatic failure. Hepatology, 4(3): 359-363, 1984.

Zanchin G, Rigotti P, Vassanelli P & Battistini L: Blood-brain barrier after portacaval shunt (PCS) in the rat: plasma influence on the altered amino acid transport. In: Hossmann KA & Klatzo I (eds) Cerebrovascular transport mechanisms. Springer Verlag, Berlin: New York, p 137, 1983.

Zaslavsky BY, Mestechkina NM, Miheeva LM & Rogozhin SV: Measurement of relative hydrophobicity of amino acid side-chain by partition in an aqueous two-phase polymeric sysytem: hydrophobicity scale for non-polar and ionogenic side-chains. J Chromatography, 240: 21-28, 1982.

Zieve L & Nicoloff DM: Pathogenesis of hepatic coma. Annual Preview of medicine, 24: 143, 1975.

Zieve L., Olsen R.L. Can hepatic coma be caused by a reduction of brain noradrenaline or dopamine? Gut, 18,688, 1977.

Zieve L: Hepatic encephalopathy: Summary of present knowledge with an elaboration on recent developments. In: Popper H & Schaffner F (eds) Progress in liver diseases. Grune & Stratton, New York, Chapter 18, p 327-341, 1979.

Zieve L: Coma production with NH_4^+: synergistic factors. Gastroenterology, 78: 327, 1980a.

Zieve L: Synergism among toxic factors and other endogenous abnormalities in hepatic encephalopathy. In: Brunner G & Schmidt FW (eds) Artificial liver support, Spring-Verlag, Berlin: Heidelberg: New York, p 18-24, 1980b.

Zieve L, Onstad GR, Doizaki WM, Zimmerman WR & Palm SR: High brain concentration of phenylalanine, tryptophan and methionine do not cause coma in rats or dogs. Gastroenterology, 79(5): 1070, 1980.

Zieve L. The mechanism of hepatic coma. Hepatology 1(4):360-365, 1981.

Zieve L., Doizaki W.M., Zieve F. Synergism between mercaptans and ammonia or fatty acids in the production of coma: A possible role for mercaptans in the pathogenesis of hepatic coma. J. Lab. Clin. Med., 83,16, 1974.

Zimmerman L, Baldesten A, Bergström J & Fürst P: Isotachophoretic separation of middle molecule peptides in uremic body fluids. clin Nephrol, 13(4): 183-188, 1980.