PLASMA AMMONIA VALUES IN NORMAL AND IN PATHOLOGICAL STATES OF CATTLE, DOGS, AND RATS

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THESIS

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ABSTRACT

PLASMA AMMONIA VALUES IN NORMAL AND IN PATHOLOGICAL STATES OF CATTLE, DOGS, AND RATS

By

Frances A. Kennedy

A cation exchange resin technique for plasma ammonia determination was evaluated in experimental and clinical cases of nutritional and hepatic disturbances in cattle, dogs, and rats. This technique's reliability was determined by recoveries and plasma vs serum samples, and by measuring the effect of 5 days of storage. Vitamin E deficient rats fed silver acetate and cod liver oil had a three-fold increase in plasma ammonia in comparison to rats not fed cod liver oil. Rats with higher ammonia values also had hepatic degeneration. After a 6-day starvation cows fed a high-protein ration had higher plasma ammonia values than cows fed a low-protein ration. Plasma ammonia values in 4 dogs and 1 cat with hepatic disturbance were higher than control subjects. Plasma ammonia values were lower 30 minutes after feeding calves a diet containing up to 1% urea. Elevated plasma ammonia as determined by the cation exchange technique was a reliable indicator of hepatic injury in animals.

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Frances A. Kennedy

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	5
Biochemistry of Ammonia Metabolism	5
Hepatic Encephalopathy	11
Reye's Syndrome	17
Portosystemic Anastomosis	19
Ammonia Metabolism in Malnutrition	23
Ruminant Ammonia Metabolism	29
Methods of Blood Ammonia Determination	35
Summary	38
OBJECTIVES	40
MATERIALS AND METHODS	41
Blood Ammonia Determination	41
Recoveries and Serum vs Plasma	45
Vitamin E Deficiency in Rats	46
Bovine Starvation	47
Dietary Urea	47
Clinical Cases	48
Histopathologic Technique	48
RESULTS	50
Blood Ammonia Determination Technique	50
Vitamin E Deficient Rats	50
Bovine Starvation	54
Dietary Urea	59
Clinical Cases	61
Case 1	61
Case 2	65
Case 3	65
Case 4	66
Case 5	66
DISCUSSION	68
Blood Ammonia Determination Technique	68
Vitamin E Deficient Rats	69
Bovine Starvation	70
Dietary Urea	71
Clinical Cases	72

Page

SUMMARY. 74

BIBLIOGRAPHY 75

LIST OF TABLES

Table		Page
1	Recoveries of ammonia standards added to human plasma.	51
2	Ammonia levels in simultaneously run human plasma and serum samples	52
3	Changes in ammonia levels in plasma samples held at -20 C	53
4	Plasma ammonia levels in Sprague-Dawley rats with experimentally induced liver damage	55
5	Effect of 6 days of starvation on bovine plasma ammonia levels.	58
6	Plasma ammonia levels (µg/100 ml) of calves fed increasing amounts of urea.	60
7	Blood urea nitrogen levels (mg/100 ml) of calves given dietary urea.	62
8	Laboratory results of patients suspected of having hepatic disease	63
9	Blood ammonia concentrations (µg/100 ml) of patients suspected of having hepatic disease	64

LIST OF FIGURES

Figure		Page
1	Biochemical pathways of ammonia metabolism.	8
2	Possible sites of ammonia toxicity in the brain	16
3	Effect of ammonia on malate-aspartate shuttle in the brain	24
4	Light micrographs of liver of a rat given a vitamin E deficient diet, silver acetate, and cod liver oil	57
5	Electron micrographs of liver of a rat given a vitamin E deficient diet, silver acetate, and cod liver oil	57

INTRODUCTION

Ammonia (NH_3) has long been suspected of having a key role in the development of central nervous system (CNS) disturbance in relation to metabolic disease. The liver acts as a detoxification center for many of the body's metabolites, including NH_3. It is during episodes of liver failure that patients experience signs referable to the brain, giving rise to the term hepatic encephalopathy (HE). A fairly constant laboratory finding in these patients is elevated blood NH_3 levels (Bessman and Bessman, 1955; Schenker et al., 1974; Snyder, 1978; Walker and Schenker, 1970). Experimental confirmation of ammonia's part in pathogenesis of HE has been thwarted by the unreliability of clinical laboratory determinations of blood NH_3 values. Much work, therefore, has been aimed at developing an accurate technique for blood NH_3 determination which would be convenient for use in a clinical laboratory.

Most recently, attention has turned to the possible role of NH_3 in the pathogenesis of Reye's syndrome. As early as the 1920's, a syndrome of fatty degeneration of the liver accompanied by severe neurological disturbance was reported (Brain et al., 1929). It was not until much later that the disease and its possible pathogenesis were more fully characterized (Reye et al., 1963). The relationship between the brain and liver having been recognized, the body's

metabolites (most specifically NH_3) have been considered the possible cause of acute brain swelling seen in Reye's syndrome (Huttenlocher et al., 1969). Experimental data are being accumulated to support this hypothesis (Altenau and Kindt, 1977; Kindt et al., 1977).

While Reye's syndrome is associated with acute liver failure, portocaval shunts (PCS) are responsible for a chronic deviation in the body's normal detoxification system. Portocaval shunts are being reported with increasing frequency in veterinary literature (Beech et al, 1977; Cornelius et al., 1975; Prouty, 1975; Rogers et al., 1977; Vitums, 1961; Vulgamott, 1979). Through dietary control or surgical correction (Strombeck et al., 1977), these patients are sometimes maintained so that permanent CNS damage due to elevated intracranial NH_3 does not occur. On the other hand, a PCS is sometimes created surgically in an effort to control ascites (Keefe et al., 1961). Following such surgery the clinician must deal with the same problems seen in a naturally occurring PCS. Experimentally produced PCS have been created to evaluate the metabolic alterations in liver and brain resulting from altered liver blood flow (Hindfelt et al., 1977; Silen et al., 1957; Starzl et al., 1973).

Consideration of ammonotelic metabolism should not be limited to the brain, however. Ruminants can use non-protein nitrogen (NPN) sources in lieu of more expensive dietary protein. It has been shown that microbial use of NH_3, from urea, for protein synthesis in the rumen can be an efficient method of providing the ruminant with essential amino acids (Hogan and Weston, 1970; Loosli, 1949; Smith, 1975). Optimum levels of NPN have been sought to achieve maximum bacterial protein metabolism (Satter and Slyter, 1974; Slyter et al., 1973). With the overzealous use of NPN in the ruminant diet, however,

urea toxicity can result. Hydrolysis of urea to NH_3 has been shown to proceed more rapidly than microbial utilization of NH_3 for protein synthesis (Bloomfield et al., 1960). The consequence of excess urea intake, therefore, is an elevation of rumen NH_3 (Ciszuk, 1973; Hillis et al., 1971) and subsequent hyperammonemia (Hogan, 1961). Treatment of urea toxicity has been aimed at prevention of hyperammonemia, with some studies being done on the possible effects on bovine reproductive performance (Word et al., 1969).

Some research has also been carried out on the possible effect of NH_3 toxicosis during rehabilitation of malnourished individuals (Stevens et al., 1975). Fairly distinct neurological signs have been produced in dogs given protein deficient diets (Stewart and Platt, 1968). Neurological signs have been seen in children recovering from kwashiorkor. Some clinicians have suspected NH_3 as a contributor to these signs. Again, however, the difficulty and unreliability of blood NH_3 determinations have limited studies of these cases (Balmer et al., 1968). A balanced amino acid replacement has been found to be very important in recovery of these patients. An arginine-deficient diet given either therapeutically (Heird et al., 1972) or experimentally (Morris and Rogers, 1978) has resulted in CNS disturbance associated with hyperammonemia.

The central problem in all these areas of study has been a lack of a trustworthy method of measuring blood NH_3. Microdiffusion techniques were introduced in the 1930s (Conway, 1935) but were found to be cumbersome and variable in their results (Acland and Strong, 1968). Techniques in which blood is deproteinized have also been in use clinically (McCullough, 1967). This technique requires whole blood, however, so the values obtained are uniformly high. An enzymatic

method of blood NH_3 determination has recently been developed (DaFonseca-Wollheim, 1973). This technique is quite expensive, making it of limited value as an experimental technique. A cation exchange resin technique has shown the most promise as a reliable means for determining plasma NH_3 on large numbers of blood samples (Dienst, 1961; Hutchinson and Labby, 1962; Miller and Rice, 1963).

Since no research in the area of NH_3 metabolism could be carried out without a serviceable blood NH_3 determination technique capable of handling large numbers of samples, study into the area of laboratory technique was highly warranted. It was for this reason that work was begun using blood samples from various clinical and experimental cases of liver damage and dietary alteration. The cation exchange resin technique was used for plasma NH_3 determination.

LITERATURE REVIEW

Much recent literature has been devoted to metabolism of NH_3 and its effect on the pathogenesis of disease. This review outlines those articles most pertinent to this research, with particular attention paid to NH_3 metabolism in the CNS, ruminant utilization of NH_3, and recent developments in blood NH_3 determination techniques.

Biochemistry of Ammonia Metabolism

Ammonia is an essential nutrient necessary for the biosynthesis of protein. As important as it is in normal metabolism, its excess can cause overloading of the processes of protein and nucleic acid synthesis, resulting in accumulation of free NH_3 (Committee, 1979). Ammonia belongs to the class of weak electrolytes which exist in part as undissociated molecules. The degree of ionization of NH_3 governs its behavior in chemical reactions, adsorption to surfaces, and penetration of membranes (Visek, 1968). Mammals take in dietary protein in excess of that needed for protein synthesis. Americans, for example, use amino acids for 10 to 25% of caloric needs. During amino acid degradation, NH_3 is released in the intestinal lumen (Committee, 1979). The NH_3 is absorbed and travels via the portal circulation to the liver, where it is removed from the blood. Even when 70% of the liver has been removed, mammals can tolerate higher than normal blood NH_3 concentrations due to the efficiency of the liver's NH_3 detoxification mechanism (Visek, 1968). Most catabolism of absorbed amino acids takes

place in the liver (Lehninger, 1970), in which various biochemical pathways work to remove free NH_3 from the tissue (Figure 1).

While the liver is working to remove NH_3 from the blood, the kidney is usually a net NH_3 producer. The major source of NH_3 produced in the kidney is thought to be glutamine (Lewis, 1976). In acidosis, glutaminases in renal epithelium release NH_3 from glutamine. The NH_3 molecule then accepts hydrogen ions to form ammonium ion (NH_4^+), which is excreted in the urine. Alkalosis associated with hypokalemia (often induced by diuretics) increases net NH_3 formation (Committee, 1979). In hypokalemia, extracellular alkalosis combined with intracellular acidosis favors entry of NH_3 into cells. This augments the pathogenesis of hepatic coma seen in hyperammonemia (Lewis, 1976). Normal intracellular pH in skeletal muscle is 7.0 compared with 7.4 for extracellular pH. This pH difference favors diffusion of NH_3 into the cell so that intracellular NH_3-NH_4^+ levels are 2.5 times those seen extracellularly (Visek, 1968). This diffusion rate has been calculated, using red blood cells (RBC), to be 3.6×10^{-11} moles NH_3/sec for each RBC. Once inside the cell, the NH_3 is instantly ionized to the poorly diffusible ion NH_4^+ (Klocke et al., 1968).

In the liver, α-ketoglutarate is the ultimate acceptor of amino groups from most of the other amino acids. It can also accept NH_3 in the following reaction:

$$\alpha\text{-ketoglutarate} + NAD(P)H + H^+ + NH_4^+ \xrightarrow[\text{dehydrogenase}]{\text{glutamic acid}} \text{glutamic acid} + NAD(P)^+ + H_2O$$

The reaction is pulled to the right, so that NH_3 is trapped. Because of this, normally only small amounts of NH_3 can exist in the presence of α-ketoglutarate. This reaction is reversible, however, so that NH_3

can either be bound or released. Ammonia is more effectively trapped in the rapid reaction:

$$\text{Glutamic acid} + NH_3 + \text{ATP} \xrightarrow[\text{synthetase}]{\text{glutamine}} \text{glutamine} + \text{ADP} + \text{Pi}$$

The reaction equilibrium lies well to the right, resulting in low levels of free NH_3.

The glutamine molecule has no net charge and so can move freely through membranes. It is the only molecule other than glucose that can freely cross the blood-brain barrier in substantial quantities. Once inside a cell, glutamine can release glutamic acid and NH_3. These molecules are less permeable because at physiological pH only 1% of total NH_3-NH_4^+ exists as the uncharged, diffusible NH_3 molecule (Committee, 1979).

While glutamine synthesis is the major fate of exogenous NH_3, urea formation is responsible for the final excretion of NH_3. If NH_3 is given intravenously to a dog, urea can be formed at a rate of 2 mg of nitrogen per kilogram per minute (Committee, 1979). Elevated blood NH_3 levels will normally stimulate urea synthesis, but excessive NH_3 can inhibit respiration and substrate utilization in the liver (Katunuma et al., 1966). Cases of urea cycle enzyme deficiency have been reported. Congenital arginosuccinate synthetase deficiency in a dog resulted in hyperammonemia because of inability of the liver to convert NH_3 from the intestines to urea for excretion (Strombeck et al., 1975).

Of particular interest is metabolism of NH_3 in the brain. Not all enzymes necessary for the various steps of NH_3 detoxification are present in the brain (Figure 1). This, combined with the fact that hyperammonemia is most dramatically evidenced in CNS signs, makes

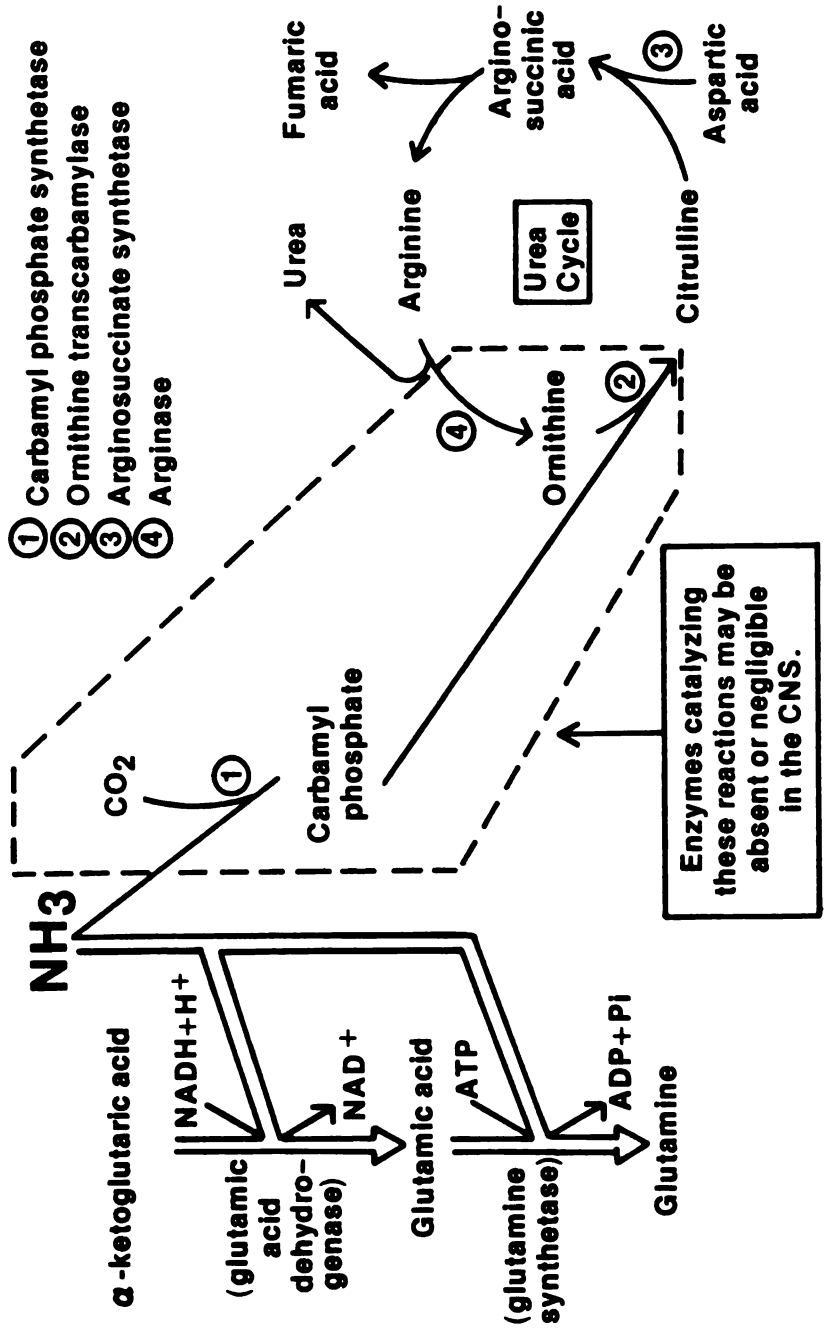


Figure 1. Biochemical pathways of ammonia metabolism.

discussion of NH_3 biochemistry in the brain most important. Brain NH_3 levels have been shown to be elevated in excitatory states, such as convulsions. On the other hand, depressed states of brain neuronal activity, such as anesthesia or hibernation, have lower brain NH_3 concentrations. It has also been shown that brains taken from immature rats (less than 3 weeks old) have twice the tissue NH_3 concentration as seen in mature rat brain (Tsukada, 1972). Elevated tissue NH_3 levels, however, may be interpreted as either cause or effect of altered brain function in these conditions. More precisely, clinical signs of hepatic encephalopathy have been thought to be related to elevation of blood and brain NH_3 levels. These signs, including asterixis (flapping tremor), decerebrate rigidity, hyperpnea, and coma, have clinically been related to dysfunction in the base of the brain. Because of this, investigation has been carried out to analyze the dynamics of the brain's handling of excess NH_3 levels and to locate specific areas of the brain most altered by toxic effects of NH_3 (Schenker et al., 1967).

Glutamine synthetase activity is localized in the extramitochondrial cytoplasm of glial cells of the brain (Committee, 1979). In hyperammonemic states the synthesis of glutamine has priority over other energy-consuming reactions that bind free NH_3 in the brain (Tsukada, 1972). Glutamic acid dehydrogenase levels are elevated in brain tissue from experimental cases of hyperammonemia. As would be expected, elevated blood NH_3 reduced brain glutaminase levels, and so suppressed the release of free NH_3 from glutamine. When hyperammonemia was combined with subacute liver injury due to carbon tetrachloride, however, glutamic acid dehydrogenase levels decreased and glutaminase levels increased in brain. It would appear, therefore, that normal

liver function is necessary to maintain these enzymes at proper levels in the brain for NH_3 detoxification (Kisfaludy, 1975).

The molecular kinetics of the hyperammonemic state have been studied using brain slices in varying tissue media. When brain slices are incubated in a medium with high levels of potassium, NH_3 accumulation decreases. This suggests that NH_4^+ and K^+ compete for the same ion transport across brain cell membranes. Ammonia formation is also depressed by high levels of glucose in the medium. This has been interpreted as due to either less proteolysis with more available glucose or an NH_3 binding mechanism (Tsukada, 1972). The effect of excess NH_3 on brain oxygen consumption has had varying results. Oxygen consumption has been reduced in brain slices by high levels of NH_3 in some laboratories (Tsukada, 1972; McKhann and Tower, 1961), while another laboratory found no alteration in brain O_2 consumption with high NH_3 concentrations in the tissue media (Schenker et al., 1967).

Alteration of intracellular pH has been blamed for the changes in biochemical reactions in brain tissue. It has been shown, however, that if carbon dioxide tensions are kept relatively stable in brain tissue, intracellular acidosis seen in NH_3 intoxication is offset by transport of basic NH_3 molecules into the cells (Hindfelt and SiesjöI, 1971). As with O_2 consumption, adenosine triphosphate (ATP) levels in brain tissue with elevated NH_3 concentrations have been found either to be unchanged (Hindfelt and SiesjöII, 1971) or decreased (Schenker et al., 1967). These results still leave the possibility of a small, isolated, critical area of ATP depletion which may be immeasurable due to its relation to the rest of the brain chemistry.

Hepatic Encephalopathy

The most devastating effects of deranged NH_3 metabolism are generally seen in CNS disturbances. While there is still controversy as to its pathogenesis, hepatic encephalopathy (HE) is believed by most workers to be caused by abnormal NH_3 metabolism. One researcher has even found a case of post-cirrhotic encephalopathy in Shakespearean literature. Sir Andrew Aquecheek (Twelfth Night; Or What You Will, 1602) was noted for his alcoholism and deranged intellect and said:

> Methinks sometimes I have no more wit than a Christian or an ordinary man has; but I am a great eater of beef, and I believe that does harm to my wit.

Shakespeare seems to have unknowingly constructed a case of hepatic cirrhosis complicated by protein intoxication (Summerskill, 1955). It is known today that digestion of meat releases NH_3, which is absorbed and carried via the portal vein to the liver. In cirrhosis, not only is the liver unable to metabolize NH_3 to form urea, but portal vessels shunt around the fibrotic liver and carry high levels of NH_3 into the blood (Snyder, 1978).

While abnormal liver function is a prerequisite for the development of HE, certain conditions can precipitate coma in individuals with compensated liver function. Any protein digestion in the intestinal tract results in NH_3 release. While ingested meat protein is the most common source, gastrointestinal hemorrhage and constipation also increase amount and duration of protein availability in the intestinal lumen (Snyder, 1978). Kidney failure may also contribute when blood urea levels become elevated. Urea can freely diffuse from the blood into the intestinal lumen. There urea is hydrolyzed by bacteria to release NH_3 for absorption. This NH_3 must then be cycled back to the liver for urea formation (Committee, 1979). If liver

function is impaired, blood NH_3 levels become markedly elevated. Inborn defects in the urea cycle may also result in intermittent CNS signs resembling HE. These defects include congenital arginosuccinic aciduria, citrullinuria, arginase deficiency, congenital lysine intolerance, and rare cases of carbamyl phosphate synthetase or ornithine transcarbamylase deficiency (Lewis, 1976).

Hepatic encephalopathy can present with a variety of clinical manifestations. In humans, dementia (mental deterioration) is the earliest sign, with the patient showing increased irritability, untidiness, apathy or altered sleep rhythm (Schenker et al., 1974). These subtle signs will progress to rigidity and tremor. Rare focal CNS changes, including irreversible paraplegia, have been reported (Lewis, 1976). The most characteristic clinical sign of HE is asterixis, or "flapping tremor." Here the fingers are held laterally with flexion and extension of the metacarpophalangeal and wrist joints every one to two seconds. This is usually bilateral but asynchronous (Schenker et al., 1974). If this progresses to decerebrate rigidity and coma, the prognosis is very poor. The patient's condition may improve, but usually the CNS signs progress. Hepatic encephalopathy has been categorized by the Modified Parsons-Smith Criteria as follows:

Grade 0: no abnormality detected.
Grade 1: subtle personality change, trivial lack of awareness, shortened attention span, impairment of addition and subtraction.
Grade 2: lethargy, facade if personality present, but confused, obvious personality change.
Grade 3: very confused, semistupor or somnolent, gross disorientation.
Grade 4: coma

(Snyder, 1978)

In some respects HE clinically resembles the encephalopathy seen in Wilson's disease. However, the latter is characterized by familial occurrence with abnormalities in copper metabolism (Victor et al., 1965). Also, the fact that HE patients do sometimes recover suggests a metabolic rather than a structural defect (Schenker et al., 1974).

Clinical laboratory data are variable in cases of HE. An elevated blood NH_3 value is often seen, while an exaggerated blood NH_3 elevation in response to oral NH_3 challenge is more specific (Strombeck and Gribble, 1978). Some workers feel there is a direct relationship between severity of CNS signs and blood NH_3 levels (Victor et al., 1965; Bessman and Bessman, 1955), while others feel the correlation is not so reliable (Schenker et al., 1974; Eiseman et al., 1955). Electroencephalographic changes may not be diagnostic and some liver function tests may be normal. Hypoglycemia is often seen, probably due to altered glycogen metabolism in the liver. Elevated cerebrospinal fluid (CSF) glutamine is a fairly consistent finding (Schenker et al., 1974).

Histopathologic liver lesions seen in HE will depend on the particular case, since many hepatic alterations can produce HE. Encephalopathy has been reported in connection with cirrhosis (Snyder, 1978), chronic active hepatitis (Strombeck and Gribble, 1978), and obstructive jaundice, where impairment of bile excretion of NH_3 was considered to be contributory to hyperammonemia (Meyer et al., 1980). A separate section of this review will cover anomalies of portal circulation which also cause hyperammonemia and HE. Pathological changes in the brain vary. One report outlines cases in which "bilateral foci of degeneration and astrogliosis" in the basal ganglia were the most prominent changes (Victor et al., 1965). Others report

degeneration of the white matter in HE (Hoerlein, 1971). Changes in protoplasmic astrocytes are by far the most consistently reported alteration in both human and veterinary medical literature (Schenker et al., 1974; Lewis, 1976; Strombeck et al., 1975; Hoerlein, 1971; Sherding, 1979; Beech et al., 1977). This altered cell has been designated the Alzheimer type II astrocyte. By light microscopy the cell has a pale, vacuolated, twisted nucleus with little cytoplasm. The astrocyte is thought to regulate brain extracellular fluid K^+ concentrations. It has been theorized that, since the K^+ and NH_4^+ ions have the same charge as well as approximately the same radius, elevated brain NH_4^+ may interfere with K^+ flux into and out of the astrocyte (Lewis, 1976).

By far the greatest controversy has centered on the possible pathogenesis of HE. Since the liver is capable of synthesizing cerebral stimulants as well as detoxifying cerebral depressants (Committee, 1979), the lack of the liver's normal function would ultimately result in altered brain function. So-called "liver tropic factors" are thought to be necessary for normal brain metabolism, and their lack results in the brain being more susceptible to injury (Lewis, 1976). If a brain is maintained in a brain perfusion system with no liver in the system, normal cerebral metabolic and electrical activity will cease. If, however, the nucleotides cytidine and uridine, which are normally liberated from the liver, are added, normal cerebral function is maintained (Walker and Schenker, 1970). The most durable hypothesis for the pathogenesis of HE seems to be the energy depletion theory (Bessman and Bessman, 1955). This theory proposes that α-ketoglutarate is depleted in the formation of glutamine in NH_3 detoxification. Alpha-ketoglutarate is needed in the brain's

Krebs cycle for energy production. Its use in NH_3 detoxification, therefore, blocks cerebral energy synthesis (Figure 2). This hypothesis is supported by the finding of elevated brain glutamine and low brain stem ATP and phosphocreatine in HE (Lewis, 1976). While the mechanism is controversial, most workers believe impaired energy metabolism at least contributes to some degree to the development of HE (Schenker et al., 1974; Walker and Schenker, 1970; Eisman et al., 1955).

Ammonia is not the only compound implicated in the pathogenesis of HE. Short chain fatty acids (butyric, valeric, octanoic) released by digestion of dietary fats are thought to have a direct toxic effect on neuronal synaptic membranes. Biogenic amines (octopamine, β-phenylethanolamine) from intestinal amino acid degradation are thought to act as weak neurotransmitters, blocking normal synaptic transmission (Sherding, 1979). Disturbed amino acid metabolism is also thought to play a role in HE. Normally, plasma branched-chain amino acids (valine, leucine, isoleucine) are at approximately three times the level of aromatic amino acids (phenylalanine and tyrosine). Insulin tends to keep plasma branched-chain amino acids at low levels, with glucagon having the opposite effect. While insulin's influence tends to dominate, the liver metabolizes aromatic amino acids to keep the ratio at 3:1 (branched-chain:aromatic). When liver function is disturbed, however, plasma aromatic amino acid levels rise. Because branched-chain and aromatic amino acids compete for the same transport mechanism into the brain, this allows more aromatic amino acids than normal into the brain. Aromatic amino acids are precursors for the inhibitory neurotransmitter serotonin, while branched-chain amino acids are necessary for the formation of excitatory neurotransmitter

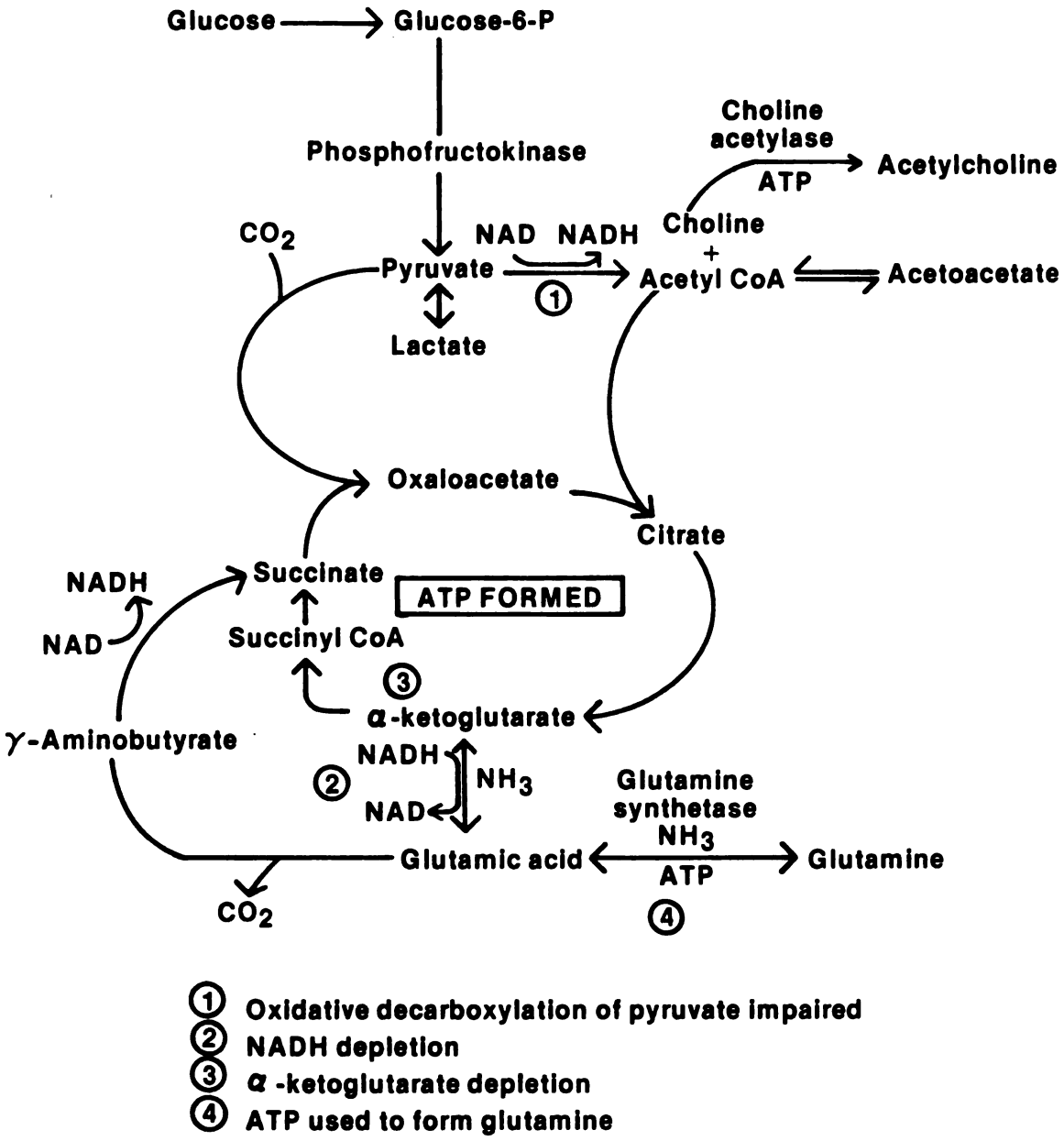


Figure 2. Possible sites of ammonia toxicity in the brain.

norepinephrine. The overall effect, therefore, is CNS depression (Strombeck and Rogers, 1978; Sherding, 1979).

Reye's Syndrome

Of recent interest is the occurrence of an acute CNS disturbance following flu-like symptoms. This disorder was designated "encephalopathy and fatty degeneration of the viscera" by Reye and his associates in 1963 but was later called simply Reye's syndrome. In most cases patients seemed to be recovering from an initial illness resembling a cold or flu, when severe vomiting began accompanied by high fever (Huttenlocher et al., 1969). The patient first was very excited, exhibiting hyperpnea and tetanic spasms followed by convulsions in severe cases. A characteristic posture was seen in these patients, with elbows flexed, legs extended, and hands clenched. Average survival time was 27 hours from the onset of symptoms. Those that survived, however, returned to normal function with no noticeable impairment of CNS function (Reye et al., 1963). After Reye's report, cases were reported from other hospitals in which an identical syndrome had been seen and attributed to ingested toxins (Elliot et al., 1963; Brain et al., 1963). Attention was then brought to a previous report in which an epidemic of six cases of the syndrome were described as "acute meningo-encephalitis of childhood", with patients ranging in age from 3 to 18 years (Brain et al., 1929).

Clinical laboratory data on these patients included low glucose levels in both blood and CSF with elevated serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT). A somewhat delayed prothrombin time has also been reported (Reye et al., 1963; Huttenlocker et al., 1969). An aid in differentiating this from other liver disorders is a normal serum bilirubin level. The

most striking and consistent clinical pathological abnormality, however, is an elevation in blood NH_3 level (Huttenlocker et al., 1969). There was a report of one case in which lumbar puncture to obtain CSF brought about a remarkable remission of signs, suggesting the possibility of elevated CSF pressure in these cases (Brain et al., 1929).

Autopsies of patients having these signs have revealed cerebral swelling grossly. Microscopically, neurons in the cerebral cortex have undergone eosinophilic necrosis, sometimes in the laminar pattern seen after convulsions of any cause (Reye et al., 1963; Huttenlocker et al., 1969; Brain et al., 1929). Edema of cerebral tissue has also been reported (Brain et al., 1929; Huttenlocker et al., 1969). In the areas of neuron necrosis, astrocyte nuclei are sometimes swollen (Huttenlocker et al., 1969), but inflammatory infiltrate is not seen (Brain et al., 1929; Reye et al., 1963; Huttenlocker et al., 1969). The liver in cases of Reye's syndrome is grossly enlarged, firm, and bright yellow and the renal cortex is slightly widened and pale (Reye et al., 1963). Microscopically, there is diffuse fatty change in the liver and the proximal convoluted tubules of the kidney (Huttenlocker et al., 1969). Some cases also have fatty change of the myocardium and pancreatic acinar cells (Reye et al., 1963). The liver does not have evidence of inflammatory infiltrate and resembles the liver of a child dying of starvation or of any disease which results in virtual starvation. It does not resemble the liver seen in acute, fulminant hepatitis, in which case the liver is usually shrunken with pronounced necrosis and inflammatory infiltrate (Huttenlocker et al., 1969).

In sorting out the clinical signs and lesions seen in Reye's syndrome, certain insight can be gained into its possible pathogenesis.

The vomiting seen at the onset may be the result of excessive CNS stimulation. Hyperpnea was initially thought to be a response to metabolic acidosis. This is accompanied by respiratory alkalosis, however, which would indicate overcompensation for any acidosis in the system. It is therefore believed that hyperpnea is due to excessive stimulation of respiratory centers in the medulla (Huttenlocker et al., 1969). Because of the consistent elevation of blood NH_3 levels, researchers have tried to reproduce Reye's syndrome by injecting intravenous ammonium acetate into experimental animals. Cats were given NH_3 along with Evans blue dye in order to visualize any extravasation of fluid from cerebral vasculature. In these experiments edema was not seen, as defined by increased tissue water (Kindt et al., 1977). When Rhesus monkeys were given intravenous NH_3, cerebral blood flow increased markedly to the point that autoregulation and response to CO_2 stimulation were lost. Grossly, cerebral vessels were engorged during the experiment. These researchers hypothesized that reduced cerebral energy metabolism and tissue lactacidosis would stimulate vasodilatation. Another possibility would be a direct toxic effect of NH_3 on vessel walls, inhibiting their normal response (Altenau and Kindt, 1977).

Portosystemic Anastomosis

Veterinary literature has devoted a great deal of discussion to the subject of anomalous anastomoses between the portal vein and the systemic circulation. Normally only about 30% of the liver's blood supply arrives via the hepatic artery, with the portal vein as the source of the remaining 70%. Along with its purpose of supplying nutrients to the liver, the portal vein carries the by-products of bacterial and enzymatic degradation of ingested material from the

intestine to the liver for detoxification. The colon in particular produces large quantities of NH_3 which must be converted to urea in the liver. When the portal vein bypasses the liver and diverts its flow into the systemic circulation, blood NH_3 levels become markedly elevated, especially after a meat meal. This NH_3 then may lead to encephalopathy not unlike that seen in liver failure, with stupor, incoordination, behavioral changes and convulsions the most often reported signs (Cornelius et al., 1975). Clinicians consistently find an unusually small liver in these animals (Sherding, 1979; Vulgamott, 1979; Cornelius et al., 1975; Prouty, 1975) when the shunt is the primary lesion. Ascites is also recognized in some cases when portal hypertension or hypoproteinemia results (Rogers et al., 1977).

Fasting blood NH_3 is usually elevated in animals with portocaval shunts (Vulgamott, 1979; Beech et al., 1977; Cornelius et al., 1975). An NH_3 tolerance test should be carried out, however, for a definitive diagnosis. Thirty minutes after oral administration of 100 mg/kg NH_4Cl, blood NH_3 values will rise 300 to 400% above fasting levels in animals with portocaval shunts (Sherding, 1979). Other clinical pathological data will vary depending on the extent of liver damage resulting from the abnormal blood supply to the liver. Slight elevations in SGPT and sulfbromphthalein retention have been seen, apparently due to chronic impairment of hepatocyte metabolism and reduced blood flow. Ammonium biurate crystals are also frequently found in the urine (Cornelius et al., 1975).

Anatomical alterations in portal circulation can be either acquired or congenital. Acquired portosystemic shunts are usually the result of obstruction of the portal vein. Accounts in veterinary

literature have described such obstruction due to peritoneopericardial herniation of the liver (Vulgamott, 1979), cirrhosis, malignant lymphoma (Vitums, 1961), and portal vein thrombosis (Beech et al., 1977). These obstructions result in portal hypertension and dilatation of normally nonfunctional communications between portal and systemic veins, known as varices. In dogs and cats these communications have been identified as the following:

1. gastrophrenic collaterals (gastric veins to phrenicoabdominal veins)
2. pancreaticoduodenal
3. splenorenal collaterals (splenic vein to left renal or gonadal veins)
4. mesenteric collaterals (mesenteric vein to left renal or gonadal vein)
5. hemorrhoidal collaterals in the sacral region
6. omental collaterals involving paraumbilical or ventral abdominal veins [often secondary to omental adhesions]

(Vulgamott, 1979)

Care must be taken in identifying one of these shunts as acquired, however, since congenital dilatation of these venous varices sometimes occurs (Thrall, 1980). Portocaval shunts have also been created surgically for treatment of chronic ascites due to heart failure in dogs (Keefe et al., 1961).

More common than these acquired shunts are those present at birth due to a congenital abnormality (Prouty, 1975; Rogers et al., 1977). In animals these anomalies include the following:

1. persistent patency of fetal ductus venosus
2. atresia of portal vein with functional collateral portosystemic shunts
3. anomalous connection of portal vein to the caudal vena cava caudal to liver
4. anomalous connection of portal vein to the azygous vein
5. drainage of the portal vein and caudal vena cava into the azygous vein

(Cornelius et al., 1975)

Histological changes in these livers are much less revealing than the gross lesions. Generally, centrolobular congestion, degeneration, and atrophy are found (Vulgamott, 1979) along with periportal fatty change and fibrosis (Beech et al., 1977; Cornelius et al., 1975). In a horse with an acquired portocaval shunt, hyperammonemia, and encephalopathy, Alzheimer type II astrocytes were found in the basal ganglia and cerebral cortex (Beech et al., 1977).

While treatment would seem a simple matter of ligating the shunting vessel, this results in acute portal hypertension and death (Sherding, 1979). Instead, a method of reducing the shunt lumen size by approximately 80% has given good results in a dog with shunting between mesenteric vessels and the caudal vena cava (Strombeck et al., 1977). If surgery is impossible, treatment is essentially the same as used in most patients where chronic hyperammonemia is a problem. This includes a low protein diet and neomycin orally to reduce intestinal bacterial flora. A synthetic disaccharide, lactulose, has also proven effective in lowering intraluminal pH. This keeps the ammonia molecule in the ionized state which is not readily absorbed (Cornelius et al., 1975).

As stated earlier, livers in animals with portosystemic anastomosis are smaller than normal. In vascular transposition experiments it was found that this is not due entirely to a reduced total blood volume into the liver. Instead, it was discovered that pancreatic hormones, especially insulin, could stimulate hepatocyte hypertrophy and hyperplasia. If, however, pancreatic venous flow is diverted from a particular liver lobe, this area will atrophy (Starzl et al., 1973; Silen et al., 1957). The effect on brain metabolism in portocaval shunts has been studied in an effort to further clarify the mechanism

of hepatic encephalopathy. Rats with surgically created portocaval shunts are much more susceptible to elevated blood NH_3 concentrations than are normal rats. These rats will develop behavioral encephalopathy at blood NH_3 concentrations which would cause very little or no behavioral change in normal animals. Biochemical analyses of the brains of these rats have given insight into the pathogenesis of this encephalopathy. In this research, an impairment of the malate-aspartate shuttle was found, resulting in reduced cerebral ATP. Glutamate is used in the detoxification of NH_3 and so is not available for its part in this cycle (Figure 3). Unlike previous results, brain α-ketoglutarate depletion did not occur when blood NH_3 levels were elevated (Hindfelt et al., 1977).

Ammonia Metabolism in Malnutrition

In the process of digestion in a normal human gastrointestinal tract, approximately 4 grams of NH_3 are produced during a 24-hour period. In addition to ingested nitrogenous nutrients, degradation of epithelial and bacterial debris contributes to the NH_3 load. It has been estimated that 15 to 30% of urea produced in the liver is eventually secreted into the stomach or small intestine and hydrolyzed to NH_3. While the NH_3 produced in the digestive tract is primarily due to the action of bacterial ureases in the colon, some is produced by mucosal urease in the stomach and small intestine. Ammonia itself may be secreted into the stomach, the diffusion regulated by differences between blood and stomach NH_3 concentrations. Once in the gastric lumen, the acidity of the contents ionizes the molecule and traps it as the poorly diffusible NH_4^+ ion. Ammonia may also be secreted by the jejunum, thus reducing blood NH_3 concentration. This is believed to be an active process in the jejunum, while NH_3 absorption is passive.

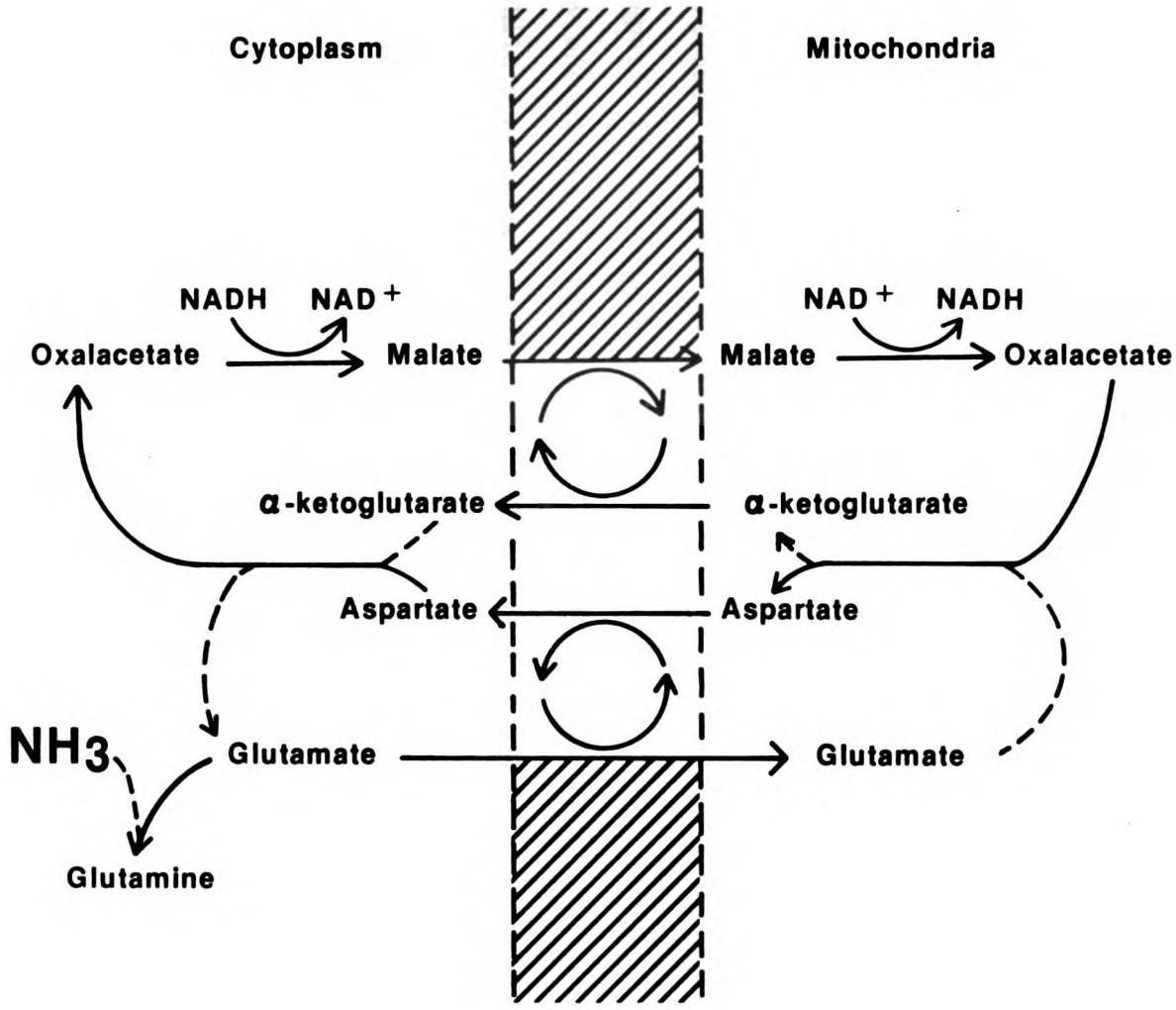


Figure 3. Effect of ammonia on malate-aspartate shuttle in the brain.

Ileum NH_3 absorption has been shown to be an active process with bicarbonate being excreted in the process. Colonic NH_3 absorption is entirely due to a concentration gradient, however, with pH variations profoundly altering the rate of absorption (Summerskill and Wolpert, 1970).

Because protein digestion and NH_3 metabolism follow interlinking pathways with carbohydrate necessary for energy in the process, it is clear that nutritional deficiencies of either protein or carbohydrate would most likely affect NH_3 utilization pathways. Protein-calorie malnutrition (PCM) is a complex syndrome which may involve total caloric malnutrition (marasmus) or deficiency of protein as the dominant problem (kwashiorkor) (Vahlquist, 1972). In kwashiorkor children have some neurological signs similar to those seen in hepatic failure (Balmer et al., 1968). Given the name "kwashi shakes" by one clinician, the signs are particularly noticeable during the child's recuperative period (Woodd-Walker, 1970). While a rhythmic twitching of the trunk and face was seen in this study, most reports list more subtle, progressive changes. The child is most often alternately irritable and apathetic (Shappley et al., 1969; Sachdev et al., 1971), followed by decreasing consciousness, stupor, convulsions, and coma (Webber and Freiman, 1974). One case study has reported two cases of kwashiorkor in which a typical "liver flap" or asterixis was seen (Balmer et al., 1968). The signs are generally found to be worse in more severe cases of malnutrition (Patel et al., 1972). Other pertinent findings include generalized edema (DeMaeyer, 1976) and hepatomegaly (Shappley et al., 1969; Webber and Freiman, 1974).

Clinical laboratory data in cases of PCM have given some information as to its effect on the body's metabolism. Elevated bilirubin, which seems to parallel severity of CNS signs, and increased SGPT indicate an effect on liver integrity in kwashiorkor (Webber and Freiman, 1974). Lowered blood glucose levels prompted a study which found that the relationship between the hypothalamus, pituitary and adrenal gland was intact (Prinsloo et al., 1974). Amino acids were low or absent in the CSF (Patel et al., 1972), with branched-chain amino acids particularly low in peripheral blood (DeMaeyer, 1976). Somewhat supportive of a link with NH_3 toxicosis was a significantly elevated CSF glutamine found in two patients with kwashiorkor in another case study (Balmer et al., 1968).

A consistent change on light microscopic examination of these cases has been a markedly fatty liver without necrosis or inflammation (Sachdev et al., 1971; DeMaeyer, 1976). Electron microscopic studies have found a phenomenon of infolding of rough endoplasmic reticulum in both liver (Webber and Freiman, 1974) and pancreas (Blackburn and Vinijchaikul, 1969) of kwashiorkor patients. Hepatocytes also have frequently contained cytoplasmic inclusions which have the fibrillar-granular appearance of alcoholic hyaline. This change has been found in many liver disorders, including acute alcoholic liver injury, Wilson's disease, chronic active hepatitis and cirrhosis (Webber and Freiman, 1974). Some microscopic alterations have also been found in the CNS of children dying of kwashiorkor. Primarily limited to the brain stem nuclei and anterior horns of the spinal cord, these changes include increased numbers of glial cells and loss of neurons (Sachdev et al., 1971). In experimental protein deficiency in pigs, brain edema was also a prominent change. While no Alzheimer type II astrocytes

were seen, in the anterior horns of the spinal cord there was an increased number of astrocytes with enlarged, vesicular nuclei. In the cerebral cortex of these pigs there was an increased diameter of astrocytic fibers (Stewart and Platt, 1968).

The suggestion of a possible hepatic origin of neurological signs seen in kwashiorkor prompted a work in which rats were fed protein-deficient diets and then challenged with NH_4Cl or amino acid injections. It was found that blood NH_3 values in deficient rats were twice those of controls before challenge as well as after either NH_4Cl or amino acid injections. In addition, the NH_4Cl caused deficient rats to become acutely ill within 10 minutes of injection, while control rats showed mild or no signs of NH_3 toxicity. Many of these rats exhibiting NH_3 and amino acid intolerance had markedly fatty livers, the severity of the fatty change somewhat paralleling NH_3 levels in blood. Neither fatty change nor blood NH_3 values correlated with the duration of protein deprivation, however. These researchers speculated that urea cycle enzymes had been sacrificed for amino acid-forming enzymes during protein deficiency (Stevens et al., 1975). In this and another study, it has been shown that animals on high protein diets develop increased NH_3 tolerance with an elevation of urea cycle enzymes in the liver (Visek, 1968; Stevens et al., 1975). Protein deficient rats, however, had a three-fold decrease in urea production (Enwonwu and Sreebny, 1970).

Enzymatic alterations in PCM have at least begun to define these deficiencies in the metabolic pathways of the brain and liver. Mitochondrolysis has been seen accompanied by a reduction in oxidative enzymes, succinate dehydrogenase, glutamic and malic enzymes (Pokrovsky, 1976; Svoboda and Higginson, 1964). These alterations seem to be

limited to immature animals, since no change in protein synthesis or oxygen uptake was found in the brains of mature rats fed protein-deficient diets (Ogata et al., 1968). In people, the extremes of dietary inadequacy and excess have been studied clinically. In patients severely depleted due to problems not involving the liver, it was found that, if a high energy diet is fed, fat is formed preferentially unless a substantial nitrogen source is also provided. This amounts to approximately 63% fat and 37% lean tissue gained by overfed adults (Elwyn et al., 1979). At the opposite extreme, obese patients placed on a liquid formula diet lost nitrogen in direct proportion to lean body mass, blood urea remaining level in these cases (Wilson and Lamberts, 1979). This nitrogen loss seems to have two sources in man. A smaller labile pool can be quickly lost without variation in a large stable pool of nitrogen. Obese people were found to be more efficient in conserving nitrogen in a fast because of a larger labile nitrogen source (Forbes and Drenick, 1979).

While total protein deficiency produces its effect on nitrogen metabolism, specific deficiency of the amino acid arginine can lead to hyperammonemia in some immature subjects. Concern first developed when babies on total intravenous feeding began exhibiting clinical signs characteristic of hepatic encephalopathy. Lethargy and decreased responsiveness in these babies progressed to twitching and frank grand mal seizures. Blood NH_3 levels in these patients were markedly elevated (823 µg/100 ml). The infusates were tested and found to contain only a negligible amount of NH_3, so this was ruled out as the source. Considering an imbalance in the amino acid mixture, supplemental arginine glutamate was given. This rapidly

reversed the CNS signs, the blood NH_3 dropping to 295 µg/100 ml. This led attending physicians to believe that the amino acid mixture used contained insufficient arginine (Heird et al., 1972).

Under experimental conditions, near-adult cats were fasted and then fed a complete meal of essential amino acids devoid of arginine (an isonitrogenous amount of alanine was substituted). Within two hours the animals became hyperesthetic and ataxic. This progressed to tonic spasms, bradypnea, and cyanosis, with one cat dying in apnea. Blood NH_3 rose during this period from 170 µg/100 ml to 1400 µg/100 ml. When the arginine was replaced with ornithine, however, no change in behavior was seen. The same experiment in adult dogs produced no such results. These workers concluded that the cat without dietary arginine is unable to synthesize ornithine at a rate sufficient to maintain the urea cycle for NH_3 disposal (Figure 1). Free arginine is rapidly depleted from liver stores, and incoming amino acids provided in the present study inhibited release of arginine from tissue stores (Morris and Rogers, 1978). It can only be speculated that the same mechanism was involved in the human infants.

Ruminant Ammonia Metabolism

From a nutritional standpoint, NH_3 is probably most important in the ruminant. Because of the unique chemical and physical makeup of the ruminant digestive tract, NH_3 can be used to synthesize protein. Ammonia produced in normal protein digestion can be recycled to the rumen for additional protein production. Proteolysis occurs rapidly in the rumen so that, almost immediately after a protein meal, rumen amino acid and peptide levels rise. Normally, nutritionally significant amounts of amino acids are not absorbed from the rumen but instead pass to the lower digestive tract for absorption (Smith, 1975).

Deamination of these amino acids occurs more slowly than proteolysis in the rumen, so that there is a three hour lag after a protein meal before rumen NH_3 rises (Blackburn, 1965). Ammonia has been shown to be absorbed from all levels of the ruminant digestive tract except the abomasum and upper small intestine (Ciszuk, 1973). Because of this, the elevated rumen NH_3 may not affect blood NH_3 for some time. Changes in rumen NH_3 were paralleled by portal blood NH_3 alterations after a two-hour lag period. Peripheral blood NH_3 was not altered, however, unless portal blood NH_3 exceeded 80 mg NH_3-N/100 ml. Above this concentration, NH_3 can overcome the liver's ability to detoxify it and it is in the peripheral blood (Word et al., 1969). Elevations in rumen NH_3, therefore, usually are reflected in blood urea nitrogen (BUN) elevations (Lewis, 1970; Ciszuk, 1973). Ammonia can be lost from the rumen by incorporation into microbial cells, direct absorption through the rumen wall, or by passage to the lower small intestine for absorption (Nolan and Leng, 1972).

Rumen NH_3 absorption takes place by simple diffusion (Lewis, 1970). After absorption, NH_3 travels via portal circulation to the liver, where it is converted to urea. This urea may either remain in the blood for possible eventual secretion back into the rumen or be secreted in saliva and so return to the rumen by that route (Hoshino et al., 1966). Researchers have measured 0.5 grams of nitrogen (N) returning to the rumen daily from the saliva (Blackburn, 1965). Levels of this salivary N in the form of urea and NH_3 tend to follow fluctuations in BUN (Houpt, 1970).

As in other parts of the body, rumen NH_3 diffusion is governed by concentration gradients as well as pH gradients. As intraruminal pH rises, more and more NH_3 is in its non-ionized form. As such, it is

more able to permeate the rumen wall (Visek, 1968). Normally, the rumen contents are acid (pH 6.5) and NH_3 absorption is dependent primarily on its concentration in rumen contents. As the rumen becomes more acid (pH 4.5), NH_3 entering the rumen from any source is rapidly ionized to NH_4^+. The pH gradient therefore favors rumen contents, acting as a trap for the NH_4^+ ion (Houpt, 1970). In this situation, however, NH_3 concentration in the rumen still favors diffusion into the blood. There is some speculation that such a low pH may alter the rumen epithelium so that, despite concentration gradients, NH_3 transport into the rumen is favored, since blood NH_3 is lower when the rumen is very acid (Hogan, 1961).

Of course, rumen pH will depend on the diet given the animal. On a natural protein diet, rumen pH is 6.5, rumen NH_3 reaches 34 mg/100 ml, and BUN peaks at 27 mg/100 ml. When urea is given as the N source, pH of rumen contents rises to 7.5. This, combined with a rumen NH_3 content of 87 mg/100 ml, facilitates NH_3 absorption into the blood, resulting in a maximum BUN of 35 mg/100 ml (Hillis et al., 1974). All changes in rumen NH_3 cannot be predicted by pH and concentration gradients alone. Studies in cattle on a concentrate diet indicate that rumen NH_3 and BUN may vary depending on the time of day. Rumen NH_3 was found to rise continually through the latter part of the night, with highest levels recorded just before the morning feeding. Rumen pH was markedly lowered during the night, with a rapid rise in early morning. Like rumen NH_3, BUN also was highest during the final part of the night. Urinary excretion of N varied throughout the day, independent of BUN. Tissue NH_3 may also vary throughout the day. All these diurnal changes affecting NH_3 levels in blood and rumen contents

may, therefore, mask any effect feeding may have on these values (Diszuk, 1973).

The key to the ruminant's utilization of NH_3 is the microbes in the rumen itself. Bacterial protein and carbohydrate were estimated to provide 750 grams of dry matter per kilogram of body weight in the ruminant (Smith and McAllan, 1973). Ammonia is the most important source of nitrogen for most bacteria and is essential for growth of some types (Smith, 1975; Commission, 1976; Allison, 1970). In cattle, urea is a commonly used source of ammonia. Urea must first be hydrolyzed by bacterial urease before it can be used for protein synthesis. If excess hydrolysis of urea occurs, NH_4^+ ions can inactivate urease in a type of feedback mechanism (Wall and Laidler, 1953). In one study, only 20% of microbial nitrogen had ruminal amino acids as its source, while 80% was derived from NH_3. Thirty percent of rumen NH_3 is eventually recycled as bacteria are ingested by protozoa (Nolan and Leng, 1972). Studies have shown that, with urea as the total dietary source of nitrogen, all amino acids essential to the ruminant can be synthesized by rumen bacteria (Loosli et al., 1949; Nolan and Leng, 1972; Commission, 1976). Urea hydrolysis occurs at a rate of 80 mg urea-N per hour per 100 ml of rumen fluid. Since rumen bacteria can only assimilate 20 mg of NH_3-N per hour per 100 ml of rumen fluid, the balance of NH_3 must be lost to the portal circulation or to the lower digestive tract, where it may eventually be recycled to the rumen (Bloomfield et al., 1960). Urea supplementation in fact lowers the ratio of bacterial N to total N by 15 to 25% when compared to a N-free diet (McAllan and Smith, 1972). Amino acid synthesis appears to proceed most efficiently when rumen NH_3-N is between 4.5 and 5.0 mg per 100 ml of rumen fluid (Slyter et al., 1973; Miller, 1973; Satter

and Slyter, 1974). Below this level, microbial protein synthesis rises as NH_3 in the rumen rises, but more NH_3 than this does not result in increased protein synthesis (Smith, 1975).

Urea may not be necessary in the ruminant diet if protein is sufficient (Allison, 1970). While microbial protein may be synthesized from amino acids, it is necessary for the amino acids to first be broken down to release NH_3. From this point, protein synthesis proceeds as if urea had been the NH_3 source. The additional step of proteolysis and deamination, however, results in a net energy loss. This is, therefore, a less efficient method for protein production in the rumen (Hogan and Weston, 1970; Commission, 1976). As in any biochemical reaction, bacteria need an energy source for the production of protein for eventual use by the ruminant. Nutrients for ATP production must be matched with N availability in order to achieve maximum use of NH_3 (Smith, 1975). Starch and other carbohydrate sources in the ruminant diet will facilitate use of urea by providing readily available energy (Visek, 1968; Houpt, 1970). The glycolytic reaction is somewhat impeded, however, by the anaerobic environment of the rumen (Chalupa et al., 1970; Commission, 1976).

With energy available, glutamic acid dehydrogenase is the primary enzyme responsible for initial fixation of NH_3 to a carbon skeleton, α-ketoglutarate (Allison, 1970; Commission, 1976). Glutamine synthetase can then fix NH_3 to glutamic acid so that glutamine can be available as a reservoir of NH_3, which can be released by glutaminase. When urea is fed, there is an elevation of NH_3 utilization enzymes (glutamine synthetase, carbamyl phosphokinase and glutamic acid dehydrogenase), with a reduction in those enzymes that produce NH_3, namely bacterial urease. Rumen mucosa also contains enzymes for urea

hydrolysis and NH_3 utilization. Urea from the blood may be hydrolyzed by mucosal urease, the NH_3 produced most likely being pulled back into the blood (Houpt, 1970; Chalupa et al., 1970; Nolan and Leng, 1972). Ammonia may also be formed by the action of mucosal glutaminase on glutamine (Hoshino et al., 1960). The rumen, reticulum, and omasum mucosa also contains glutamic acid dehydrogenase (Hoshino et al., 1966), while rumen mucosa contains glutamine synthetase (Commission, 1976). The overall effect is such that, as rumen NH_3 rises, both rumen and plasma glutamine rise, while glutamic acid and α-ketoglutarate levels decrease. Some workers believe that urea as a total N source may result in a reduction of enzymatic activity, with NH_3 having a possibly detrimental effect on the animal as a whole (Chalupa et al., 1970; Visek, 1978).

Clinically, the greatest concern has been the possibility of overzealous use of urea in the ruminant diet with resulting NH_3 toxicosis. Conditions predisposing an animal to urea toxicity include poor quality roughage in the diet (Coombe et al., 1960; Word et al., 1969), lack of adaptation to urea, fasting before urea is fed, insufficient water, errors in feed formulation, and elevated rumen pH (Commission, 1976). Too much urea itself will raise rumen pH and so facilitate NH_3 absorption (Smith, 1975). In addition to previously discussed signs of NH_3 toxicosis, ruminants also develop rumen stasis at the onset of urea toxicity. It is not known if this is due to elevated NH_3 concentration or raised pH. The most important key to prevention of urea toxicosis is to distribute the urea uniformly through the diet so that the animal gets a slow, steady dose of urea. Ruminants can tolerate much higher total doses of urea if it is fed over a long time interval (Coombe et al., 1960). If urea overdose does

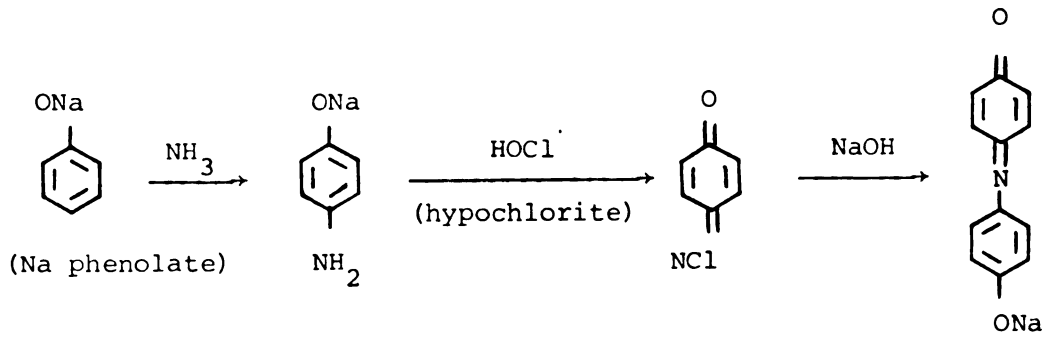
occur, recommended treatment includes large volumes of cold water to dilute the NH_3 and to inhibit ureolysis. Acetic acid or vinegar may also help by lowering intraruminal pH (Commission, 1976).

Methods of Blood Ammonia Determination

A brief review of the various methods of blood NH_3 determination is necessary to understand the advantages and disadvantages of each. A microdiffusion technique introduced in the 1930s was probably the first method by which any reproducibility of results could be obtained. The apparatus used in this technique is a glass petri dish with an inner well. Samples of blood are placed in the outer chamber and mixed with potassium carbonate. The alkalinity causes NH_3 to form a diffusible gas which passes into the inner chamber. In the inner chamber, NH_3 is absorbed into a standard acid and the resulting pH change measured by titration of barium hydroxide and visible by a color change of methylene blue and methyl red. Absorption time of 10 minutes allows 50% of blood NH_3 to be absorbed at room temperature, while 15 minutes results in 66% absorption. Results obtained by the initial work in human subjects averaged 33 µg/100 ml (Conway, 1935). A more recent study has found the microdiffusion method to be unreliable, with 65 to 144% recovery of added NH_3 standards. The primary problem was considered by these workers to be the highly alkaline conditions necessary for microdiffusion. This alkalinity results in NH_3 formation from other nitrogenous substances in plasma (Acland and Strong, 1968).

In addition to its problem with alkalinity, use of titration was thought to be less accurate than other colorimetric methods of NH_3 determination. Using the microdiffusion technique for isolation of NH_3, a solution of sodium phenolate and hypochlorite was used with

nitroprusside as catalyst (Yernberg and Hershey, 1960). The reaction proceeds as follows:



This indophenol reaction results in a soluble and stable colored product (McCullough, 1967). Optical density of this product has been found to be unchanged after 24 hours at room temperature (Ternberg and Hershey, 1960). This color reaction has also been used on whole blood samples which have been deproteinized by tungstate and sulfuric acid. Deproteinized extracts of heparinized whole blood samples can be read by direct addition of the indophenol solutions. In one report, 93 to 104% recovery of NH_3 standards was obtained, with normal human values of 72 to 84 µg/100 ml (McCullough, 1967).

A technique of enzymatic blood NH_3 determination has recently been reported (DaFonseca-Wollheim, 1973). Using plasma, blood NH_3 levels can be measured by its reaction with α-ketoglutarate, as follows:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADPH \xrightarrow[\text{dehydrogenase}]{\text{glutamic acid}} \text{glutamate}$$

$$+ NADP + H_2O$$

(Bio-Dynamics, 1977)

The final result is actually measured by the amount of NADPH oxidized. Photometrically, a reduction in NADPH is seen as a decrease in absorbance. This technique has resulted in 100% recoveries of NH_3 standards with a high specificity for NH_3 (DaFonseca-Wollheim, 1973;

Bio-Dynamics, 1977). A drawback, however, is that this is a relatively expensive technique if large numbers of samples are to be handled.

A less expensive and highly reliable technique for blood NH_3 determination is the cation exchange resin technique. Using this method, blood NH_3 values from normal subjects averaged 40 µg/100 ml less than with the Conway microdiffusion technique. This is because there is no need for extreme alkalinization of blood in the resin technique, so that plasma constituents are not converted to NH_3 (Dienst, 1961). The resin adsorbed with sodium and potassium is able to exchange Na^+ and K^+ ions for NH_4^+, so that NH_3 is adsorbed to the resin (Hutchinson and Labby, 1962). The NH_4^+ is then eluted from the resin with dilute NaOH. The indophenol color reaction can then be used for final NH_3 determination, producing a dark blue complex that is read at a maximal absorbance of 640 mµ on a spectrophotometer. After washing with ion-free water, the resin with NH_4^+ adsorbed can be held up to three hours at room temperature without appreciable change in final NH_3 value (Miller and Rice, 1963). Controls and standards run at the same time as samples can account for any NH_3 present in room air (Hutchinson and Labby, 1962). Recoveries using this method were reported to be between 93 and 98% in one study (Acland and Strong, 1968). This technique is also quite flexible, which is important in an experimental situation. Smaller amounts of blood than initially recommended can be used simply by reducing volumes of reagents used (Roller et al., 1970; Kirkpatrick et al., 1972; Jacquette et al., 1974).

As important as the technique used for NH_3 determination is the handling of blood samples. Immediately after blood is drawn, it is

important to put samples on ice so as to slow any NH_3 production from plasma components. When this is done, only slight elevation in NH_3 content results after 30 minutes (Ternberg and Hershey, 1960). Arterial samples have been found to be consistently higher in NH_3 content than venous samples, most likely due to tissue uptake of NH_3. Because of this, arterial NH_3 should not be equated with venous NH_3 either clinically or experimentally (Davidovich et al., 1977). Plasma is preferred over both whole blood and serum for determination of NH_3. Plasma has been found to be more stable for longer periods, so that less NH_3 is generated in samples while standing. Disodium ethylenediaminetetraacetate (EDTA) is the preferred anticoagulant, since heparin is frequently contaminated with NH_3 (Ratliff and Hall, 1979). It is also important to prevent hemolysis of samples, since 2% hemolysis has reportedly increased NH_3 concentration by 3 to 5%, supposedly due to NH_3 release from RBCs (Ternberg and Hershey, 1960). An extremely valuable use of NH_3 determination is the NH_3 tolerance test. Patients fasted for 12 hours are given 100 mg/kg of NH_4Cl (maximum 3 grams) orally with 20 to 50 ml of warm water. Blood samples are drawn before dosage and 30 minutes after NH_4Cl. Normal animals tested in this way had no significant rise in blood NH_3, while animals with portosystemic shunts had 344 to 409% increases in blood NH_3. In many liver disorders this is more specific than fasting NH_3 or BSP retention in pinpointing hepatic abnormalities (Meyer et al., 1978).

Summary

Ammonia is important in both normal and pathological states of people and domestic animals. Researchers have studied the pathways of NH_3 metabolism in hopes of better understanding its effect on

normal body function. While NH_3 has been implicated in the pathogenesis of many diseases, it is also recognized as a necessary nutrient. Research has been impeded, however, by variability in results of blood NH_3 determinations. This research was carried out to establish a reliable blood NH_3 determination technique which would be practical for experimental and clinical laboratory use. Experimental as well as clinical cases of liver injury and altered nutritional states were used as sources of blood samples.

OBJECTIVES

The objectives of this research were:

1. To establish a reliable technique for plasma NH_3 determination.

2. To determine normal plasma NH_3 levels in species available for sampling.

3. To determine plasma NH_3 changes in experimentally produced liver disturbance.

4. To follow plasma NH_3 changes during the period in which calves were being acclimated to dietary urea.

5. To use the cation exchange resin technique of plasma NH_3 determination on blood samples from cases of hepatic disease at the Veterinary Clinical Center, Michigan State University.

MATERIALS AND METHODS

Blood Ammonia Determination

The cation exchange resin technique was used on all samples obtained for this research. Referring to previous works using this technique (Miller and Rice, 1963; Jacquette et al., 1974), modifications were made on the original method to facilitate its use in these experimental conditions. All glassware was rendered NH_3-free by routine washing followed by a 30-minute soak in 0.1 N NaOH. The glassware was then rinsed 4 times in NH_3-free water (H_2O) and dried in a plastic basket in an oven. Glassware was then wrapped in plastic for storage. All H_2O used in this experiment was considered to be as NH_3-free as possible after being distilled and recently passed through a deionizer resin.[a]

Dowex 50W-X12 resin 200-400 mesh, hydrogen ion form, sulfonic acid type[b] was used. Phosphate buffer (0.2 M, pH 7.4) was prepared by dissolving 27.22 gm potassium dihydrogen phosphate (KH_2PO_4)[c] in NH_3-free H_2O, adjusting the final volume to 1 liter. The pH of the

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[a]CL5 Deeminizer Water Demineralizer, Crystalab, Hartford, CT.

[b]Dowex 50W-X12 Cation Exchange Resin, 200-400 Mesh, Hydrogen Form, Sulfonic Acid Type, Bio-Rad Laboratories, Richmond, CA.

[c]Potassium Phosphate Monobasic (Crystals), Mallinckrodt Chemical Works, St. Louis, MO.

resulting solution was adjusted to 7.4[d] with 20% sodium hydroxide (NaOH). The 20% NaOH solution was prepared by adding 20 gm NaOH[e] to NH_3-free H_2O and adjusting the final volume to 100 ml. A solution of 0.1 N NaOH was prepared by weighing 4.0 gm NaOH and adding NH_3-free H_2O to 1 liter. Fifty grams of resinwere then washed 3 times with 500 ml portions of 0.1 N NaOH by mixing continuously with a stir bar and magnetic stirrer,[f] 15 minutes for each wash. Between all washes the resin was allowed to settle and the supernatant was aspirated. The resin was then washed 3 times in the same manner but using 500 ml portions of NH_3-free H_2O. This was followed by 3 washes, each using 300 ml of phosphate buffer. The resin was then washed again 3 times with 500 ml portions of NH_3-free H_2O, the supernatant was aspirated, and then the resin was stored in an amber glass bottle under 400 ml of NH_3-free H_2O at 4 C. This procedure allowed adsorption of K^+ and Na^+ ions to the resin in exchange for H^+ ions.

Ammonia stock standard (200 µg/ml) was prepared by weighing 94.3 mg of dried ammonium sulfate[g] and adding NH_3-free H_2O to a 100 ml volume. This solution was stored in a plastic bottle at 4 C and was considered stable for 6 months. Working standards were prepared as follows: 100 µg/100 ml - 0.5 ml stock solution, add NH_3-free H_2O

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[d]Digital Ionalyzer, Model 501, Orion Research, Inc., Cambridge, MA.

[e]Sodium Hydroxide Pellets, Mallinckrodt, Inc., Paris, KY.

[f]Model SP-A1025B Stir Plate, Thermolyne Corp., Dubuque, IA.

[g]Ammonium Sulfate Crystals, Matheson, Coleman and Bell Mfg. Chemists, Norwood, OH.

to 100 ml; 200 µg/100 ml - 1.0 ml stock solution, add NH_3-free H_2O to 100 ml; 300 µg/100 ml - 1.5 ml stock solution, add NH_3-free H_2O to 100 ml. These solutions were also stored at 4 C in plastic bottles but were only stable for 1 month.

A phenol color reagent solution and alkaline hypochlorite solution were used to obtain the color reaction. The phenol color reagent was prepared by dissolving 5 gm of phenol (C_6H_5OH)[h] and 25 mg of sodium nitroprusside [$Na_2(NO)Fe(CN)_5 \cdot 2H_2O$][i] in NH_3-free H_2O and adjusting the final volume to 500 ml. This solution was stored at 4 C in an aluminum foil-covered bottle. The aluminum foil was used to protect the reagent from light, the solution being stable for 1 month. Alkaline hypochlorite solution was made with 2.5 gm NaOH, 4 ml of commercial grade bleach (sodium hypochlorite 5.25%)[j] and addition of NH_3-free H_2O to 500 ml. This solution was stored at 4 C in a plastic container and was stable for 6 months.

Resin tubes were made up before blood samples were drawn so as to expedite the process of adsorbing plasma NH_3 to the resin. Washed resin was pipetted into 15 ml graduated glass-stoppered centrifuge tubes so that 1 ml of a H_2O-resin solution contained 0.3 ml of resin. A tube was prepared for each blood sample with 4 additional tubes for a blank and 3 standards. Unless otherwise indicated, blood samples were collected into sterile tubes containing disodium EDTA.[k] Samples

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[h]Phenol Crystals, J. T. Baker Chemical Co., Phillipsburg, NJ.

[i]Sodium Nitroprusside (Practical), Eastman Organic Chemicals Distillation Products Industries, Rochester, NY.

[j]Big Chief Bleach (Sodium Hypochlorite 5.25%), Patterson Laboratories, Inc., Detroit, MI.

[k]Vacutainer Brand Evacuated Glass Tube (7 ml, Disodium Edetate), Becton, Dickinson and Company, Rutherford, NJ.

were immediately placed on ice. As soon as possible, the tubes were centrifuged for approximately 10 minutes, at which time plasma was separated from cells by pipetting. For each day's determination, 1 blank and 3 standards were run. Each resin tube had added either 0.5 ml of NH_3-free H_2O, 0.5 ml of each of the 3 previously prepared working NH_3 standards, or 0.5 ml of test plasma.

After solutions were added, the resin tubes were stoppered and then agitated for 1 minute on a vortex mixer.[1] After the resin was allowed to settle, the supernatant was aspirated. Three milliliters of NH_3-free H_2O was then added and the tubes were again agitated for 1 minute. The resin was then allowed to settle and the supernatant aspirated. The wash with 3 ml NH_3-free H_2O was repeated twice more or until the supernatant was free of foam to assure removal of plasma proteins. Final volume was then adjusted to 1.5 ml with NH_3-free H_2O. At this time plasma NH_3 was adsorbed to the resin. One milliliter of 0.1 N NaOH was then added to each tube and the tubes vortexed for 1 minute. After the resin was allowed to settle, 1 ml of the resulting supernatant was removed to a NH_3-free 7 to 10 ml glass test tube. This supernatant contained NH_3 which had been removed from the resin by competitive adsorption of Na^+ ions.

To the tube containing the 1 ml of supernatant, 2 ml of the phenol color reagent was added, followed by 2 ml of alkaline hypochlorite. Tubes were then capped[m] and mixed by inversion. After mixing, tubes

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[1]Vortex-Genie Mixer, American Hospital Supply Corporation, McGraw Park, IL.

[m]Parafilm "M" Laboratory Film, American Can Company, Greenwich, CT.

were placed in a 37 C water bath for 15 minutes. Tubes were then removed from the bath and mixed by inversion, and the solution was placed in 12 x 75 mm glass cuvettes. All tubes were then read after zeroing with an NH_3-free H_2O blank on a spectrophotometer[n] set at 640 mμ wavelength. Optical density (OD) of standards and samples was corrected (OD-C) by subtracting the OD of the NH_3-free H_2O blank which had been run through the resin procedure. Concentration of NH_3 was calculated in μg per 100 ml by the following formula:

$$\text{concentration unknown} = \frac{\text{concentration standard x OD-C unknown}}{\text{OD-C standard}}$$

So that the benefit of all standards could be gained, the concentration of a given standard was divided by its OD-C. The result was called the F value of that standard. The Fs of all standards were averaged and the resulting factor was multiplied by the OD-C of the unknown to obtain its concentration. Unless otherwise indicated, the above procedure was used on all samples to be discussed.

Recoveries and Serum vs Plasma

All recoveries were run on plasma samples from the same human subject (FK). The procedure used was the same as for all other samples. Identical plasma samples were placed in 4 resin tubes, 0.5 ml each. To the first tube, nothing was added, while to the other 3 tubes, 0.5 ml of each of the previously prepared working standards was added. Ammonia concentrations were then determined as usual. The value obtained from the sample to which no standard was added was subtracted

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[n]Coleman 44 Linear Absorbance Spectrophotometer, Perkin-Elmer Corporation, Oak Brook, IL.

from the NH_3 values of the other 3 tubes. The result was the recovered concentration of NH_3. When this concentration was divided by the known concentration of the standard added, a percent recovery was calculated.

In order to determine the effect of clotting on blood NH_3 values, 2 samples of blood were collected from each human subject at the same time. One sample was collected into a tube containing no anticoagulant, while the second tube contained disodium EDTA. Both tubes were placed in a 4 C refrigerator until the former sample had coagulated. Therefore, the same time elapsed between drawing of blood and addition to resin for both samples. After centrifugation, 0.5 ml of either serum or plasma was added to resin and NH_3 determination was carried out on each as usual.

Samples of plasma were saved from some days' experiments so that the effect of holding blood at -20 C could be seen. Tubes containing plasma were capped and placed in a -20 C freezer for 24 hours, 48 hours or 5 days. After the period of time had elapsed, the plasma samples were thawed and plasma NH_3 was determined as previously described.

Vitamin E Deficiency in Rats

Sixty-day-old Sprague-Dawley rats were divided into 4 groups. Group 1 was fed a control diet of corn and soybean meal with a trace mineral and vitamin mixture added. Group 2 was given the same diet with no vitamin E and .05 ppm selenium in the vitamin-mineral mixture. Group 3 was given the vitamin E and selenium deficient diet with 0.15% silver acetate added to the drinking H_2O. Group 4 was given a vitamin E and selenium deficient diet, 0.15% silver acetate in the drinking H_2O and 5% cod liver oil in the diet. After 5 months on these diets,

rats were anesthetized with ether and blood samples were drawn by cardiac puncture. The rats were then exsanguinated when the right ventricle was cut. Tissues were collected in 10% buffered formalin for histological examination. One rat from Group 3 and 2 rats from Group 4 were perfused with Karnovsky's fixative so that electron microscopic study of the liver could be done. This was accomplished by opening the chest of the anesthetized rat, infusing Karnovsky's fixative into the left ventricle, and cutting the right ventricle to allow escape of blood. The rats were thought to be fully perfused when limbs went into tonic extension. Tissue was then collected, diced into approximately 1 mm cubes, and placed in Karnovsky's fixative.

Bovine Starvation

Samples of blood for plasma NH_3 determination were collected from 5 adult Holstein cows which were used on a starvation experiment. These cows were then given no food for 6 days, while H_2O was provided ad libitum. After the 6-day starvation, blood samples were again collected and plasma NH_3 levels determined. The cows were then given either a 23% protein diet (hay, soybean meal, salt, dicalcium phosphate) or a 12% protein diet (hay, corn, salt, dicalcium phosphate). One cow given a low protein diet and 1 given a high protein diet had blood samples drawn 5 hours after resuming eating. Because the cows were to be subjected to surgery for liver biopsy, a week interval was allowed to pass with the cows being given either a high or a low protein diet before the next blood NH_3 samples were collected.

Dietary Urea

Blood NH_3 levels were determined on 10 calves before they were started on dietary urea. The calves were begun on 0.2% urea at the

age of 2 to 4 months. At weekly intervals, the urea was increased to 0.4, 0.7 and 1.0% and held at 1.0%. Blood NH_3 determinations were run at the same time weekly before the amount of dietary urea was raised. Blood urea nitrogen° was also determined for the sixth through the ninth week of the experiment. On the thirteenth week of the experiment, blood samples for NH_3 were collected before the morning feeding and 1/2 hour after feeding. The calves' diet was prepared as follows:

soy protein	1500 gm	cerelose	5750 gm
mineral mix	500 gm	urea	20-100 gm
$CaCO_3$	100 gm	vitamin mix	100 gm
cellulose	150 gm	lard	500 gm
selenium mix	10 gm	flora	5 gm
terramycin	1 gm		

Clinical Cases

Blood samples were collected from patients into sodium heparin tubes for determination of whole blood NH_3 at the Veterinary Clinical Center (VCC) clinical pathology laboratory, Michigan State University. Remaining blood was spun down and plasma was used for NH_3 determination using the cation exchange resin technique. The VCC laboratory used the technique of deproteinization with sodium tungstate and sulfuric acid as previously described (McCullough, 1967). Comparisons were made between results of the 2 techniques in both resting NH_3 and NH_3 tolerance tests.

Histopathologic Technique

Methods of fixation of tissue from rats has been described. Tissues for light microscopic examination were embedded in paraffin

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°Urea Nitrogen Colorimetric Determination Kit, Sigma Chemical Company, St. Louis, MO.

and stained with hemaxotylin and eosin (Luna, 1968). Tissue for electron microscopic study was washed in cacodylate buffer with 4.5% sucrose and then osmicated in osmium tetroxide. After dehydration, the tissue was embedded in Epon. Thick sections were stained with toluidine blue. Thin sections cut with a LKB ultramicrotome were stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope operating at 60 KV. Standard photographic technique was used to process the negatives.

RESULTS

Blood Ammonia Determination Technique

The cation exchange resin technique for blood NH_3 determination was found to be rapid and convenient for use in both solitary clinical cases and experimental procedures in which several samples were to be run at once. Recoveries of NH_3 standards from human plasma ranged from 83.1% to 96.0% on 3 separate sets of recoveries (Table 1). When plasma and serum samples were run on the same individual's blood, a highly significant difference ($p<.0001$) was found in NH_3 results. The mean value of plasma NH_3 was 15.24 µg/100 ml, while the mean serum NH_3 level was 56.94 µg/100 ml (Table 2). Holding plasma samples at -20 C did not have a significant effect on blood NH_3 values after 24 hours, the mean only increasing from 19.49 µg/100 ml to 19.72 µg/100 ml. After 48 hours under the same conditions, however, a significant rise ($p<.005$) in plasma NH_3 was seen, the mean rising from 18.50 µg/100 ml to 31.92 µg/100 ml. Freezing the plasma samples for 5 days gave results not unlike those seen at 48 hours, the mean NH_3 values rising from 16.18 µg/100 ml to 28.70 µg/100 ml (Table 3).

Vitamin E Deficient Rats

Plasma NH_3 did not rise significantly from controls in either rats given a vitamin E deficient diet or those given a vitamin E deficient diet with silver acetate in the drinking water. The means

Table 1. Recoveries of ammonia standards added to human plasma

Sample	OD	OD-C	F	µg/100 ml	Recovered µg/100 ml	Recovered %
First Recovery						
Blank	.078					
100 µg/100 ml	.139	.061	1639			
200 µg/100 ml	.197	.119	1681			
300 µg/100 ml	.253	.175	1714			
FK	.087	.009	av.1678	15.1		
FK+100 µg/100 ml	.137	.059		99.0	83.9	83.9
FK+200 µg/100 ml	.193	.115		193.0	177.9	89.0
FK+300 µg/100 ml	.241	.163		273.5	258.4	86.1
Second Recovery						
Blank	.078					
100 µg/100 ml	.130	.052	1923			
200 µg/100 ml	.187	.109	1835			
300 µg/100 ml	.242	.164	1829			
FK	.082	.004	av.1862	7.4		
FK+100 µg/100 ml	.129	.051		95.0	87.6	87.6
FK+200 µg/100 ml	.182	.104		193.6	186.2	93.1
FK+300 µg/100 ml	.222	.144		268.1	260.7	86.9
Third Recovery						
Blank	.079					
100 µg/100 ml	.130	.051	1961			
200 µg/100 ml	.190	.111	1802			
300 µg/100 ml	.248	.169	1775			
FK	.088	.009	av.1846	16.6		
FK+100 µg/100 ml	.133	.054		99.7	83.1	83.1
FK+200 µg/100 ml	.185	.106		195.7	179.1	89.6
FK+300 µg/100 ml	.244	.165		304.6	288.0	96.0

OD: optical density

OD-C: optical density corrected (OD of sample - OD of NH_3-free H_2O blank)

F: $\frac{\text{concentration of standard}}{\text{OD-C of standard}}$

av: average of F values

Table 2. Ammonia levels in simultaneously run human plasma and serum samples

Sample	Plasma NH_3 (µg/100 ml)	Serum NH_3 (µg/100 ml)	Serum NH_3-Plasma NH_3 (µg/100 ml)
FK	9.00	43.00	34.0
LS	8.00	54.10	46.1
MT	26.00	82.20	56.2
TM	17.60	44.90	27.3
JR	15.60	60.50	44.9
$\bar{X}$	15.24	56.94	
SE[a]	3.25	7.05	

[a]Standard error

$\bar{X}$: mean

Table 3. Changes in ammonia levels in plasma samples held at -20 C

	Bovine Plasma NH_3 (µg/100 ml)		Canine Plasma NH_3 (µg/100 ml)		Human Plasma NH_3 (µg/100 ml)	
	Day 0	Day 1	Day 0	Day 2	Day 0	Day 5
	29.20	33.80	16.90	24.80	13.30	20.30
	15.60	24.40	13.20	26.60	11.60	27.00
	27.30	9.40	18.80	39.00	8.30	27.00
	21.40	15.00	22.60	40.80	31.50	40.50
	9.80	24.40	18.80	40.80		
	19.50	11.30	20.70	19.50		
	9.80	20.70				
	21.40	24.40				
	11.70	11.30				
	29.20	22.50				
$\bar{X}$	19.49	19.72	18.50	31.92	16.18	28.70
SE[a]	2.40	2.46	1.32	3.83	5.21	4.24

[a]Standard error

$\bar{X}$: mean

of plasma NH_3 in Groups 1, 2 and 3 were 54.27 µg/100 ml, 62.28 µg/100 ml, and 65.67 µg/100 ml, respectively. In Group 4, however, a highly significant ($p<.005$) rise in plasma NH_3 was seen, the mean being 174.01 µg/100 ml (Table 4). These rats had been given a vitamin E deficient diet, silver acetate in the drinking water, and 5% dietary cod liver oil. On light microscopic examination, livers of these rats had moderate diffuse hepatocyte vacuolization, which had not been apparent in livers of rats from the other groups (Figure 4). These vacuoles stained positively with oil red O, indicating that they represent fatty change of hepatocytes. Electron microscopic examination was made of the liver of 1 rat each from Groups 3 and 4. The most dramatic change was seen in the Group 4 rat, which had extensive proliferation of disorganized smooth endoplasmic reticulum (Figure 5).

Bovine Starvation

The changes in the cows' plasma NH_3 levels after 6 days of starvation were so varied it was not believed that statistical significance could be drawn from the results (Table 5). Of the 5 cows used in the experiment, 3 (7, 12, 17) had a reduction in plasma NH_3 after 6 days of starvation. The remaining 2 cows (9, 10) had elevated plasma NH_3 as compared to pre-starvation levels. Of the 3 cows in the former group, 2 were started on low protein diets after starvation (7, 12). While plasma NH_3 did rise above post-starvation levels in these two, they did not reach the NH_3 concentrations seen before starvation. Cow 17, however, was placed on a high protein diet and subsequently had a dramatic rise in plasma NH_3. This plasma NH_3 reached a level of approximately 3 times pre-starvation NH_3 and almost 8 times post-starvation NH_3 concentrations. Two of the cows (9, 10) had an elevation

Table 4. Plasma ammonia levels in Sprague-Dawley rats with experimentally induced liver damage

	Group 1[a] (µg/100 ml)	Group 2[b] (µg/100 ml)	Group 3[c] (µg/100 ml)	Group 4[d] (µg/100 ml)
	100.70	54.60	101.30	206.40
	47.00	92.90	37.50	187.60
	38.60	41.90	58.20	75.00
	52.00	85.60		300.20
	47.00	36.40		135.10
	40.30			93.80
				234.50
				159.50
$\bar{X}$	54.27	62.28	65.67	174.01
SE[e]	9.50	11.44	18.82	26.30

[a]Group 1 = control

[b]Group 2 = vitamin E deficient

[c]Group 3 = vitamin E deficient with silver acetate

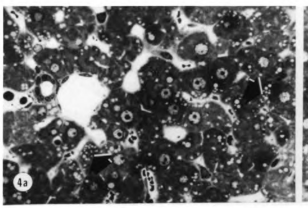
[d]Group 4 = vitamin E deficient with silver acetate and cod liver oil

[e]Standard error

$\bar{X}$: mean

Figure 4. Light micrographs of liver of a rat given a vitamin E deficient diet, silver acetate, and cod liver oil. (a) Fine vacuolization of cytoplasm is present in periportal hepatocytes (arrows). X40. (Epon, toluidine blue) (b) Large fat vacuoles are present in some hepatocytes (arrows). X40. (Epon, toluidine blue)

Figure 5. Electron micrographs of liver of a rat given a vitamin E deficient diet, silver acetate, and cod liver oil. (a) Note fat vacuoles in cytoplasm (arrow). X15,150. (b) Note the large amount of disorganized smooth endoplasmic reticulum (arrows). X27,450.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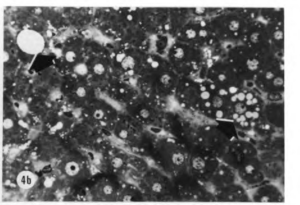
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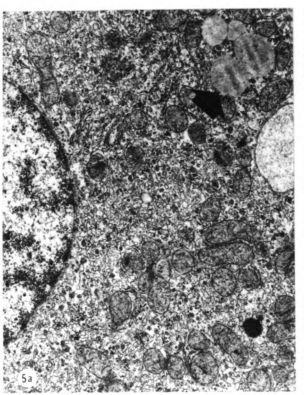
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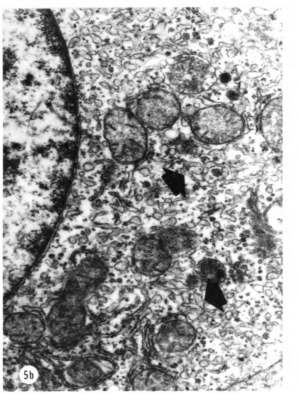
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Table 5. Effect of 6 days of starvation on bovine plasma ammonia levels

Cow No.	Before Starvation (µg/100 ml)	After Starvation (µg/100 ml)[a,b]	After Feeding Resumed (µg/100 ml)	
			5 hr	1 wk
7	56.30	10.80		14.00
9	7.80	13.40	19.10	7.70
12	36.50	17.90		28.70
10	14.40	32.30	62.70	52.70
17	64.10	25.40		201.40
$\bar{X}$	35.82	19.96		60.90
SE[c]	11.08	3.95		35.91

[a]After starvation, 7, 9 and 12 were given 12% protein diet

[b]After starvation, 10 and 17 were given 23% protein diet

[c]Standard error

$\bar{X}$: mean

of plasma NH_3 after 6 days of starvation. The one fed a 12% protein diet (9) had a further rise in plasma NH_3 5 hours after beginning the diet, while the NH_3 dropped to approximately pre-starvation level 1 week later. The second cow (10) had an approximate doubling of the post-starvation NH_3 5 hours after beginning the 23% protein diet. One week later, after continuing the high protein diet, the plasma NH_3 was still high as compared with both pre- and post-starvation values.

Dietary Urea

When calves were begun on 0.2% dietary urea, there was only a mild elevation in their plasma NH_3 concentrations (Table 6), the mean increasing from 28.79 µg/100 ml to 32.67 µg/100 ml. Contrary to expectations, however, a highly significant drop ($.0001<p<.01$) in plasma NH_3 was seen in the subsequent weeks, when urea in the feed was raised to 0.4%, 0.7%, and finally 1.0%. From a control mean NH_3 value of 28.79 µg/100 ml, the means of plasma NH_3 dropped to 16.40 µg/100 ml at 0.4% urea, 19.49 µg/100 ml at 0.7% and finally 15.55 µg/100 ml when the diet contained 1.0% urea. After the calves were maintained on this 1.0% urea diet, the plasma NH_3 values rose closer to and above control concentrations, with means for the 10 calves ranging between 20.61 µg/100 ml and 42.04 µg/100 ml. There was a noticeable drop ($.05<p<.1$) in plasma NH_3 from a mean of 42.04 µg/100 ml before the morning feeding to 25.33 µg/100 ml 1/2 hour after feeding. All previous samples had been drawn after the calves' morning feedings.

During the seventh week of the experiment, when the calves had had the most significant drop ($p<.0001$) in plasma NH_3 as compared with controls, a highly significant decrease ($p<.005$) in BUN was also seen

Table 6. Plasma ammonia levels (µg/100 ml) of calves fed increasing amounts of urea

Week	0	4	5	6	7	8	9	13	13
Urea %	0.0	0.2	0.4	0.7	1.0	1.0	1.0	1.0[a]	1.0[b]
1	36.70	66.50	30.20	29.20	27.10	21.80	30.00	25.90	37.80
2	25.70	35.10	20.70	15.60	16.30	30.90	13.10	23.90	23.90
3	42.20	27.70	9.40	27.30	7.20	36.40	33.70	89.60	17.90
4	27.50	12.90	17.00	21.40	14.50	25.40	26.20	35.90	21.50
5	16.50	25.80	11.30	9.80	10.80	41.80	11.20	25.90	17.90
6	27.50	24.00	11.30	19.50	21.70	21.80	24.40	43.80	25.90
7	27.50	31.40	7.50	9.80	14.50	12.70	7.50	101.50	8.00
8	29.30	25.80	18.90	21.40	9.00	18.20	15.00	21.90	35.80
9	36.70	44.30	26.40	11.70	21.70	36.40	22.50	28.70	35.90
10	18.30	33.20	11.30	29.20	12.70	18.20	22.50	23.30	28.70
$\bar{X}$	28.79	32.67	16.40	19.49	15.55	26.36	20.61	42.04	25.33
SE[c]	2.54	4.56	2.41	2.40	1.99	3.02	2.71	9.21	3.01

[a]Preprandial samples

[b]1/2 hour postprandial samples

[c]Standard error

$\bar{X}$: mean

(Table 7). The mean BUN dropped from 12.0 mg/100 ml to 8.8 mg/100 ml, and thereafter returned to levels approximating those seen during the sixth week.

Clinical Cases

Blood samples were available from 1 feline and 4 canine patients suspected of having hepatic disease. For all patients, a control was run along with the patient's blood in either resting NH_3 or NH_3 tolerance tests. Results obtained at the VCC laboratory were subsequently compared with those obtained using the cation exchange resin technique. All but 1 patient (Case 4) were eventually sent for postmortem examination to the Animal Health Diagnostic Laboratory (AHDL), Michigan State University. In the interest of space, only clinical and laboratory data considered pertinent to this research will be discussed. Clinical pathology results found on presentation of each case have been summarized in Table 8, while blood NH_3 concentrations are compared in Table 9.

Case 1

An 11-year-old male German Shepherd dog had a history of inappetence and weight loss of 2 weeks' duration. On the day of examination, the owners had noticed dark, bloody, odorous diarrhea and dark yellow urine being passed by the dog. Clinical laboratory data indicated anemia, leukocytosis and low BUN with elevation of SGPT, alkaline phosphatase (AP), and bilirubin. Hepatic injury was further evidenced by NH_4-biurate crystals and bilirubin in the urine. Resting plasma NH_3 was 4 times the control concentration and rose 1.5 times 30 minutes after NH_4Cl was administered in an NH_3 tolerance test. On postmortem

Table 7. Blood urea nitrogen levels (mg/100 ml) of calves given dietary urea

Week	6	7	8	9
Dietary Urea %	0.7	1.0	1.0	1.0
1	11.0	7.0	12.0	11.0
2	11.0	9.0	12.0	11.0
3	13.0	11.0	14.0	14.0
4	11.0	7.0	12.0	11.0
5	10.0	8.0	12.0	10.0
6	17.0	9.0	13.0	12.0
7	14.0	9.0	12.0	11.0
8	10.0	9.0	6.0	10.0
9	10.0	8.0	17.0	12.0
10	13.0	11.0	13.0	11.0
$\bar{X}$	12.0	8.8	13.3	11.3
SE[a]	0.7	0.4	0.6	0.4

[a]Standard error

$\bar{X}$: mean

Table 8. Laboratory results of patients suspected of having hepatic disease

	Case 1[b]	Case 2[c]	Case 3[b]	Case 4[b]	Case 5[b]
PCV (%)	29	19.1	49	32.8	35.2
WBC (/µl)	54,400	46,000	13,600	26,600	8,600
Total protein (gm/100 ml)	7.2	6.6	6.4	4.8	5.2
BUN (mg/100 ml)	8	22	9	7	8
Glucose (mg/100 ml)	98	155	113	75	96
SGPT (IU/l)	100	153	1770	23	20
Alkaline phosphatase (IU/l)	330	19	1870	740	---
Bilirubin (mg/100 ml)					
-total	3.4	---	2.4	1.1	---
-direct	2.6	---	1.6	0.5	---
-indirect	0.8	---	0.8	0.6	---
BSP retention (%)			17.5	23.5	0.5
Urine - NH_4 biurate crystals	+	-	-	a	-
- bilirubin	3+	-	3+	a	-

[a]Not run

[b]Canine patient

[c]Feline patient

Table 9. Blood ammonia concentrations (µg/100 ml) of patients suspected of having hepatic disease

		Resin Technique	VCC Technique
Case 1[c]	Resting	149.7(34.5)[a]	160(50)
	30 minutes[b]	234.1	284
Case 2[d]	Resting	97.9(57.6)	146(84)
Case 3[c]	Resting	41.9(12.7)	29(43)
	30 minutes	85.5(14.6)	127(36)
Case 4[c]	Resting	103.4(11.9)	160(80)
Case 5[c]	Resting	11.9(11.9)	70(80)
	30 minutes	49.7(6.0)	120(60)
	60 minutes	15.9(6.0)	100(60)

[a]All control sample results are given in parentheses after patients' results

[b]Time after NH_3 tolerance test dose given

[c]Canine patient

[d]Feline patient

examination, a diagnosis of chronic active hepatitis was made, with necrosis of hepatic parenchyma, macrophage and neutrophil infiltrate, and moderate to severe periportal fibrosis.

Case 2

A 9-year-old castrated male Siamese cat had a history of generalized seizures of 1 day's duration. On physical examination, the liver was found to be enlarged. The cat was anemic with leukocytosis, elevated blood glucose, and increased SGPT. Feline leukemia test results were positive in this cat. Resting blood NH_3 was 1.5 times that of control. After 3 weeks of hospitalization, the cat died and a postmortem examination was performed. Perivascular edema was evident in the brain. Approximately half of the hepatic parenchyma was displaced by malignant lymphocytes, and there were also areas of necrosis in the liver. The kidney, lymph nodes, and intestine were also infiltrated, but not to the extent that the liver was involved. A final diagnosis of lymphosarcoma was made.

Case 3

A 10-year-old male Husky dog had a history of polyuria, polydipsia, and gastrointestinal distress. On physical examination, the liver was considered small. Significant laboratory data included increased SGPT, AP, and bilirubin. Retention of BSP was 17.5% on the day of admission but dropped to 4% 1 week later after treatment at the VCC. Bilirubin was also present in the urine in high amounts. Resting blood NH_3 was 3 times the control level and rose 100% 30 minutes after NH_4Cl administration. On histological examination of the liver, a diagnosis of subacute periportal hepatitis was made, with mild infiltration of fibroblasts and lymphocytes.

Case 4

A 7-year-old spayed female Schnauzer dog had a history of seizures following recovery from anesthesia. On admission, the dog was semi-conscious and lapsed into coma while hospitalized. Laboratory data showed mild anemia, leukocytosis, and hypoproteinemia with slightly decreased BUN and increased AP. Retention of BSP was 23.5% after 30 minutes, but CSF analysis was normal. Resting blood NH_3 was 8 times the control concentration. Euthanasia was performed at the owner's request, but permission was not given for postmortem examination.

Case 5

A 3-month-old male Bouvier des Flandres dog had a history of apparent blindness after recovery from anesthesia, during which respiratory arrest occurred. The referring veterinarian reported that 3 of the 4 puppies from this litter had some evidence of anesthesia intolerance. Electroretinogram, ophthalmological examination, and CSF analysis were normal, as were all other clinical laboratory data. Because of the history of anesthesia intolerance in this puppy and its siblings, an NH_3 tolerance test was run to evaluate liver function. Resting blood NH_3 equaled the control, but NH_3 rose by a factor of 4 times the resting level 30 minutes after NH_4Cl administration. Sixty minutes after NH_4Cl, however, the plasma NH_3 had nearly returned to normal. The owners requested euthanasia and a postmortem examination was performed. Conclusive lesions were not found, although focal vacuolization of the optic nerve was seen.

In almost all cases the technique used by the VCC laboratory resulted in higher levels of whole blood NH_3 than were found when the cation exchange resin technique was used on plasma. The results on whole blood ranged from 1.06 to 10 times the results obtained using

plasma and the resin method. In only 1 sample (Case 3, resting sample) did the cation exchange resin technique result in a higher value of plasma NH_3 as compared to the VCC levels (1.4 times).

DISCUSSION

Blood Ammonia Determination Technique

The cation exchange resin technique for plasma NH_3 determination was found to be reliable and convenient when used on multiple samples or single samples. Although 100% recoveries were not obtained, the use of standards run through the same procedure concurrently was believed to compensate for any loss of NH_3 left on the resin. Also, a blank run with each set of samples accounted for any variability in room air NH_3 or other contamination which may have occurred from day to day.

Plasma samples are generally preferred when running blood NH_3 determinations (Ratliff and Hall, 1979), but many laboratories still use serum or whole blood data. The highly significant increase in serum NH_3 concentration when paired blood samples were used, however, indicated that plasma would be the preferred sample. The fact that samples were prepared in precisely the same manner eliminated the possibility of handling artifact as the cause of increased NH_3. Previous studies using serum have allowed prolonged storage before serum separation, which could have accounted for increased serum NH_3 (Jaquette et al., 1974). In the present work, however, it would appear that simply the process of coagulation resulted in a generation of NH_3 in the sample. Caution should be exercised, therefore, in interpreting serum NH_3 results, since incomplete coagulation, impaired

coagulation, or even possibly the volume of the sample drawn could conceivably create a false impression of blood NH_3 concentration.

In the present study, significant alterations in plasma NH_3 levels were not present after storage at -20 C for 24 hours. Such handling could not generally be recommended, however, since previous reports have indicated 30 minutes as the recommended maximum time between venipuncture and adsorption to resin (Ternberg and Hershey, 1960). The convenience of the resin method is that once plasma NH_3 has been adsorbed and the resin washed, tubes can be held up to 3 hours at room temperature without appreciable elevation in NH_3 results (Miller and Rice, 1963). Other workers have gone so far as to say that NH_3 is stable on the resin indefinitely after thorough washing (Hutchinson and Labby, 1962). Although such leeway may be appreciated in a clinical setting, in the interest of accuracy the most rapid determination possible is recommended. As in experimental procedures, simultaneously run standards and controls can increase reliability of results.

Vitamin E Deficient Rats

A series of insults to the livers of Sprague-Dawley rats were carried out in order to hopefully produce measurable hyperammonemia. Surprisingly, vitamin E deficiency and silver acetate in the drinking H_2O did not impaire hepatic function enough to cause significant elevation of plasma NH_3. It was only when the animals were further stressed by adding 5% cod liver oil to the diet that a dramatic rise in plasma NH_3 was seen. While no brain lesions characteristic of hyperammonemia were seen, livers of Group 4 rats were more fatty than normal, as evidenced by oil red O-positive vacuoles in hepatocyte cytoplasm.

Electron microscopic study of 1 liver specimen from this group had pronounced proliferation of smooth endoplasmic reticulum with loss of normal organization. Several reports have been made of vitamin E deficiency in rats in which hepatocyte mitochondria were enlarged with bizarre shapes and fragmentation of cristae (Svoboda and Higginson, 1963; Djaczenko et al., 1969; Lantos et al., 1973). These changes suggested impaired respiratory function of the mitochondria. Endoplasmic reticulum was most often affected with vacuolar dilatation (Svoboda and Higginson, 1963), with disorganization of normal architecture. This disorganization was reportedly due to loss of stability of the endoplasmic reticulum membrane due to the absence of the antioxidant effect of vitamin E (Lantos et al., 1973). In a study of mitochondrial membranes in particular, vitamin E deficiency resulted in the production of defective membranes in excessive quantities (Frigg and Rohr, 1976). In the present experiment, it would appear that dietary cod liver oil taxed the limited amount of antioxidant available so that formation of defective lipoprotein membranes occurred. In an attempt to compensate for the weak endoplasmic reticulum membranes, greater amounts of these membranes were produced. This random proliferation of endoplasmic reticulum resulted in the disorganized array of membranes in the hepatic cytoplasm. This most likely resulted, among other things, in impaired transport of NH_3 within the hepatocyte, preventing its incorporation into the urea cycle. As a result, NH_3 was allowed to build up in the blood.

Bovine Starvation

While the cows' plasma NH_3 concentrations were varied after 6 days of starvation, there was an overall reduction in mean NH_3 levels.

This presumably was due to absence of a dietary nitrogen source for maintaining resting plasma NH_3. Since the cows were all subjected to liver biopsy after the post-starvation samples were drawn, it was thought that at least some of the elevation in plasma NH_3 seen 5 hours after feeding was resumed was due to liver trauma and overall stress to the animal. One week later, NH_3 values were all lower than controls in cows fed a low protein diet. The two cows given a high protein diet, however, exhibited dramatic increases in plasma NH_3. Of particular interest is Cow 17, which had a voracious appetite, devouring all food immediately upon its being offered. Since all samples were drawn at the same time after the morning feeding, her 201.4 µg/100 ml plasma NH_3 would seem to represent an intolerance of dietary protein. This phenomenon has been experimentally produced in protein deficient rats. After protein starvation, the rats became intolerant of oral NH_4Cl and amino acids, having exaggerated rises in plasma NH_3 (Stevens et al., 1975). This is believed to be due to a decline in urea cycle enzymes in the liver of these rats (Enwonwu and Sreebny, 1970). In the present case, although the cow did not have clinical signs consistent with NH_3 toxicosis, she did have quite significant hyperammonemia.

Dietary Urea

When calves were fed nutritional levels of urea, a predictable rise in plasma NH_3 was seen when the urea was initially introduced at 0.2% of the diet. This was followed, however, by a highly significant decrease in plasma NH_3 over the next 3 weeks as urea concentration was gradually increased to 1.0%. This is indeed a surprising reaction, since it would be expected that the calves would take some time to adapt themselves to dietary urea. A drop in BUN was also experienced when dietary urea was raised from 0.7 to 1.0%.

When cows were fed a concentrate diet, the rumen NH_3 rose during the latter part of the night, with highest levels before the morning feeding. Blood urea nitrogen was also highest at this time (Ciszuk, 1973). Another consideration is that at lower intraruminal NH_3 concentrations, the rate of microbial protein synthesis rose as rumen NH_3 rose (Smith, 1975). Readily available carbohydrate in the diet also facilitated bacterial use of NH_3 for protein synthesis by providing needed energy (Houpt, 1970). The calves in this study were fed a concentrate diet which provided sufficient energy for rapid protein synthesis. At the morning feeding, as rumen NH_3 was elevated by dietary urea, microbial protein synthesis may have been stimulated sufficiently to result in an overall drop in rumen NH_3. This, combined with the normal diurnal variation in rumen NH_3, probably caused a significant drop in intraruminal NH_3. This drop may have produced a shift in the concentration gradient so that both plasma NH_3 and urea were drawn into the rumen. Such a drop then could be seen in a reduced postprandial plasma NH_3 and BUN.

Clinical Cases

Use of the cation exchange resin technique for plasma NH_3 determination in veterinary clinical cases is particularly convenient because of the small amount of blood needed. Since only 0.5 ml of plasma is ultimately needed, 1.0 to 1.5 ml of blood was sufficient for this method. When dealing with very small dogs and cats where multiple diagnostic tests may have to be run, this may be a critical factor.

There was a considerable discrepancy between results obtained at the VCC and in the experimental laboratory, the VCC results generally being higher. This was most likely due in part to the different

conditions under which the samples were run. In a clinical laboratory, if urine or fecal samples are being handled in the same vicinity as NH_3 determinations, this may result in NH_3 contamination and falsely high values. Also, since whole blood was used in the clinical laboratory, the NH_3 released from RBC contents would also tend to result in higher values (Ternberg and Hershey, 1960). In Cases 1, 2 and 3, in which liver disturbance was confirmed by histological evaluation of the liver, the clinician should at least have been suspicious of impaired hepatic function from plasma NH_3 results. Both Cases 4 and 5 also had abnormally high plasma NH_3 levels. It is interesting to note, however, that this was only apparent in Case 5 after use of the NH_3 tolerance test (Meyer et al., 1978). Since Case 4 was lost to followup histopathological evaluation of the liver, these results could not be confirmed.

SUMMARY

A cation exchange resin technique for plasma ammonia determination was evaluated in experimental and clinical cases of nutritional and hepatic disturbances in cattle, dogs, and rats. This technique's reliability was determined by recoveries and plasma vs serum samples, and by measuring the effect of 5 days of storage. Vitamin E deficient rats fed silver acetate and cod liver oil had a three-fold increase in plasma ammonia in comparison to rats not fed cod liver oil. Rats with higher ammonia values also had hepatic degeneration. After a 6-day starvation cows fed a high-protein ration had higher plasma ammonia values than cows fed a low-protein ration. Plasma ammonia values in 4 dogs and 1 cat with hepatic disturbance were higher than control subjects. Plasma ammonia values were lower 30 minutes after feeding calves a diet containing up to 1% urea. Elevated plasma ammonia as determined by the cation exchange technique was a reliable indicator of hepatic injury in animals.

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