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TABLE OF CONTENTS

PART I. THE EFFECT OF FACTORS INFLUENCING PROTEIN METABOLISM ON TISSUE GLUTAMINE LEVEL

INTRODUCTION

In recent years considerable interest has been manifest in the role of amides in nitrogen metabolism of animals. This has in part been motivated by the discovery that amides appear to be involved in the metabolism of proteins in rapidly growing plants and germinating seeds, in part by the recognized function of glutamine as a growth-stimulating substance for many microorganisms and in part by the demonstration that glutamine is a required nutrient for the growth, in vitro, of certain types of animal cells. Free glutamine has been found in appreciable quantities in every animal tissue investigated. This universal distribution, coupled with the knowledge that considerable energy is released with the rupture of the amide bond, has led to the speculation that glutamine must serve important functions in the metabolism of the organism.

Some of the specific functional roles that have been suggested for glutamine are: a storage depot for free ammonia which is toxic to most organisms, even in relatively low concentrations; (a means of detoxifying ammonia;) an intermediate in the formation of amino acids and other nitrogen containing compounds; a coenzyme for some reactions; supplying energy for the synthesis of proteins, i.e., the formation of the peptide bond; as a source of urinary ammonia to conserve fixed base; in the synthesis of urea.

The purpose of this investigation was to further elucidate the functions of amides in animal protein metabolism. In particular, it was proposed: to further improve analytical procedures for the determination of glutamine; to determine the effect of hormones, known to be involved

in controlling protein metabolism, upon the glutamine content of certain animal tissues, i.e., thyroid-active substances, cortisone, and anterior pituitary growth hormone; to study the nature of the effect of cortisone upon protein metabolism and its possible relationship to glutamine. Any light that could be shed upon these functions would be of immeasurable value in understanding the very complex picture of protein synthesis and degradation.

REVIEW OF THE LITERATURE

The literature on glutamine to 1947 has been adequately reviewed by Archibald (1,2). Tigerman (3) summarized the publications appearing between this date and 1950. For the sake of brevity this review will be primarily concerned with publications appearing since 1950. Reference will be made to papers covered by the above mentioned reviews only when they particularly pertain to this investigation.

I. The Determination of Glutamine

The methods available for glutamine assay can be classed under 4 general types: (a) measurement of ammonia liberated from the amide position by either enzymic, acid, or base hydrolysis, (b) measurement of CO_2 liberated by the specific bacterial glutamic acid decarboxylase, (c) decrease in activity with nitrous acid or ninhydrin following ring closure to pyrrolidone-carboxylic acid, or (d) microbiological assay utilizing differences in growth of the organism. The advantages and disadvantages of some of these methods have been described by Archibald (4). Only the more recent publications will be considered here.

Wiss (5) has outlined microbiological methods for the determination of glutamine, other amino acids, and vitamins of the B complex which utilize some lactic acid bacteria and fungi, such as Phycomyces blakesleeanus, and varieties of Neurospora. He claimed production of a growth effect which can be expressed by a standard curve. Most other microbiological methods previously described have not differentiated

between glutamic acid and glutamine.

Mardashev and Mamaeva (6) have described a method whereby glutamine, glutamic acid, asparagine, and aspartic acid can be determined quantitatively. They separated the acids from their amides by adsorption of the former on Al_2O_3 which had been treated with HCl. The glutamic acid and glutamine were determined by measuring the CO_2 liberated upon addition of the specific bacterial glutamic acid decarboxylase extracted from Clostridium welchii, strain SR-12 or BW. 21. The organism was grown on 2% solid agar media and the enzyme extracted with acetone from a water suspension of the cells.

Astrup and Munkvad (7) have also utilized extracts from Cl. welchii, strain SR-12 for the determination of glutamine and glutamic acid in plasma.

Westall (8) reported the isolation of pure glutamine from selected fractions of beetroot extract by use of synthetic ion-exchange resins.

A number of paper partition chromatographic procedures have been suggested for the estimation of glutamine. These procedures give good qualitative results but have not lent themselves satisfactorily to routine quantitative analyses. The method of Block (9) has been used by several to give fair results.

II. Glutamine metabolism.

A. Synthesis

Considerable work has been done relative to the biological synthesis of glutamine. In a recent paper Elliott (10) gave the equation for the synthesis of glutamine as:

Glutamate + ATP + NH_3 = glutamine + ADP + phosphate

He stated that the number of enzymes involved is not known, but suggested that γ-glutamyl phosphate might be an intermediate which could react with ammonia to form glutamine liberating inorganic phosphate. The intermediate has not been demonstrated. The system is specific toward L-glutamate and ATP, while NH_2OH or hydrazine will replace NH_3. These findings are in agreement with earlier reported work of Bujard and Luethardt (11), Elliott (12), and Speck (13, 14). In disagreement with these investigations, Mardashev and Lestrovaya (15) report that rat-liver tissue in oxygen or nitrogen atmosphere at 30-7° and pH 8 or 6 produced no glutamine from glutamic acid and NH_4Cl. Glutamic acid and asparagine gave good yields and glutamic acid and aspartic acid gave small yields of glutamine. ATP had no effect on the reaction. They suggested a condensation of the amino acid and the amide with intramolecular hydrogen transfer, rearrangement, and cleavage to the new amide and acid. Lichtenstein et al. (16) reported an enzyme in sheep brain which synthetized glutamine from glutamate and NH_4Cl. It required ATP and was strongly inhibited by ADP. This enzyme also catalyzed a reaction between α-methyl glutamic acid and NH_4Cl. They assumed the product formed to be α-methyl glutamine. Luschinsky (17) described a glutaminase enzyme present in human placenta. The enzymic activity was greatest in early stages of pregnancy when fetal development was most rapid. Based on the order of magnitude of enzyme activity human placenta is on a par with human kidney in which glutamine metabolism has long been recognized as having an important functional role. The activity of the enzyme was increased by the addition of phosphate.

B. Glutamine and Urea Formation.

The possible role of glutamine in urea formation has been the subject of a number of recent investigations. Kamin and Handler (18) determined the maximum rates of urea production from a number of amino acids in dog by a continuous infusion technique. They also determined cellular permeability to these amino acids in liver, skeletal muscle, kidney, and brain. They found that glutamic and aspartic acids do not enter liver and muscle cells, cerebrospinal fluid, or brain, whereas glutamine readily enters all these tissues. Greatest rates of urea formation followed infusion of arginine, glutamine, and a casein hydrolyzate. They state that the rate of urea formation from NH_3 is probably greater than that from arginine. These relative rates of urea formation did not correspond with rates of deamidation or with rates of urea formation with these amino acids studied in vitro by other investigators. As the rate of urea formation when glutamine or a casein hydrolyzate was added was increased above that encountered from arginine infusion, these authors suggest the existence of an additional mechanism for the formation of urea which does not involve arginase. They postulate that glutamine or some derivative of it may actually constitute a common intermediate through which the nitrogen of amino acids may be transferred to urea. In opposition to this theory, Hirs and Rittenberg (19), as a result of studies using amide N^{15}-labeled glutamine, conclude that no specific function could be attributed to the amide nitrogen of glutamine in urea formation in liver slices. They showed that in liver slices ammonia nitrogen is much more efficiently used for urea formation than amino nitrogen of glutamic acid, aspartic acid, and alanine. They contend that aspartic

acid and glutamic acid do penetrate the liver cell.

C. Role of Glutamine in Glucose Metabolism.

Lerner and Mueller (20) show that glutamine appears to play an important role in glycolysis by Clostridium tetani. This is shown by the fact that cells from a medium deficient in iron lose the ability to ferment glucose. This ability is restored by addition of glutamine as shown by CO_2 evolution. They believe this stimulatory effect to be due to glutamine, either free or in peptide linkage. McIllwain (21,22) showed that glucose was required for glutamine metabolism in hemolytic streptococci and that glutamine markedly stimulated glycolysis as determined by CO_2 evolution. The glutamine was quantitatively decomposed to glutamic acid and NH_3. He suggests that this participation may take the form of an NH_3 transferance. McIllwain et al. (23) in later studies of this reciprocal relationship demonstrated that NH_3 was formed from glutamine during glycolysis but not from any of a number of other related compounds which were used. γ-glutamyl hydrazine inhibits this reaction and the growth of the organism in a parallel manner. Also, many substances which inhibited glycolysis inhibited NH_3 liberation from glutamine.

Dawson and Williams (24) demonstrated a marked decrease in rat liver content of both glutamine and glutamic acid which seemed to be associated with the hypoglycemic state which followed insulin administration, as this decrease could largely be prevented by injection of glucose. This decrease appeared not to be related to the release of adrenalin. In similar studies on rat brain, Dawson (25) observed a decrease in glutamic acid content of this tissue resulting from insulin hypoglycemia, but no change was observed in the glutamine

level.

D. Glutamine and the Formation of Urinary Ammonia.

For some time glutamine has been postulated as one source of the NH_3 excreted by the kidneys to conserve fixed base. Van Slyke et al. (26) in experiments with dogs, demonstrated that the amide N of glutamine was removed from blood plasma much more rapidly than it appeared in urine. The excess was enough to provide the ammonia removed from the kidney via the renal vein and 60% or more of the NH_3 excreted in the urine. Administration of glutamine to dogs in HCl acidosis markedly increased the excretion of NH_3 while HCO_3^- alkalosis resulted in a decrease in NH_3 excretion which was accompanied by a decrease in the rate of removal of glutamine from the renal blood. Davis and Yudkin (27) observed that acidosis produced an increase in kidney glutaminase activity whereas alkalosis resulted in a decrease. Rats in acidosis excreted more NH_3 than rats in an alkalosis group. Diaz et al. (28) claimed that the enzymic functions of the kidney may be impaired without any detectable kidney lesions, as a result of the absence of extrarenal hormones. In rats, hypophysectomy resulted in a decrease in kidney deaminase and glutaminase activity with resulting inability to form NH_3 to conserve fixed base. Adrenalectomy did not effect glutaminase activity but decreased deaminase and phosphatase activity.

E. Glutamine and Uric Acid Formation.

Örström and Örström (29) observed that whereas the blood glutamine level of normal persons is of the order of 10 mg.%, that of gouty patients is 1.4 mg.%. The hypoxanthine level, on the other

hand, is increased from 0.4 to 10.5 mg.%. They assume that the uric acid cycle is similar to the urea cycle except that glutamine instead of ornithine is the starting point. They point out that the formulas for the two compounds are similar, differing only in an O being replaced by 2 H atoms on C_5. They state that both glutamine and hypoxanthine are uric acid formers in birds, and assume that similar pathways are used for uric acid formation in man.

F. Glutamine in Protein Synthesis.

Tigerman and MacVicar (30) demonstrated an elevation in tissue glutamine levels in rats when placed on rations causing a negative nitrogen balance, or during inanition, both of which enhance body tissue protein catabolism. These observations suggest that glutamine may have a function in the catabolism of amino acids.

Grossowicz et al. (31) observed the presence of enzymes in cell-free extracts and resting cells of Proteus vulgaris which catalyze the exchange of hydroxylamine with the amide group of glutamine and asparagine. They suggest that these enzymes have the ability to utilize the amide bond energy for synthetic processes, e.g. peptide linkage. Phosphate, magnesium, cyanide, iodoacetate, fluoride, or ATP did not alter the rate or extent of enzyme exchange. Cell-free extracts of the organism were able to catalyze the formation of glutamohydroxamic acid from hydroxylamine and glutamic acid but ATP and magnesium were essential for the reaction. Borek and coworkers (32) showed that the minimum growth inhibiting effect of hydroxylamine to Lactobacillus casei and Proteus vulgaris could be reversed by NH_3, glutamine, and asparagine (not so pronounced in the case of L. casei with asparagine). They concluded that hydroxylamine in low concentrations

may interfere with the utilization of glutamine, NH_3, and asparagine. It may block other pathways, as well, in higher concentrations. Elliott (33) isolated a glutamine synthetase and glutamo-transferase from green peas. The transferase catalyzes the transfer of isotopic ammonia or hydroxylamine with glutamine according to the following equation:

$$RCONH_2 \text{ (glutamine)} + NH_2OH = RCONHOH + NH_3$$

A study of activities and inhibitors failed to conclusively demonstrate the existence of two enzymes and the authors hesitated to ascribe the activities to two different enzyme systems. ATP is required for both reactions. Stumpf et al. (34) described a glutamyl transferase enzyme derived from pumpkin seedlings, which likewise catalyzed the reaction between glutamine and hydroxylamine to produce glutamylhydroxamic acid and NH_3. This system required Mn^{++}, arsenate or phosphate, and ATP.

Back and coworkers (35) state that DL-N-(γ-glutamyl) ethylamine completely inhibited the multiplication of Trichomonas vaginalis when present in amounts of 135 mg. per 6 cc; 100 mg. inhibited growth to a certain extent. Addition of as little as 30 mg. of glutamine reversed this effect but 60 mg. L-glutamate had no effect. This ethylamine derivative appeared to act as a competitive antagonist of glutamine and not glutamic acid and glutamine seemed to be essential for the growth of T. vaginalis. Heathcote and Pace (36) showed that the crystalline toxic factor produced when zein is treated with NCl_3 has the structure: $CH_3S(:O)(:NH)\ CH_2CH_2CH(NH_2)COOH$. This compound is seen to have some resemblance to glutamine in chemical structure. This compound inhibits the growth of Leuconostoc mesenteroides.

A lower homolog, $CH_3S(:O)(:NH)CH_2CH(NH_2)COOH$, synthesized from L-cysteine, is ineffective as a growth inhibitor at comparable concentrations, showing that the toxic effect is not specifically dependent upon the grouping around the S. Glutamine reverses the toxic effect of this compound on L. mesenteroides.

Mueller and Miller (37) observed that glutamine was able to replace, in part, certain glutamine-containing peptides occurring in tryptic digests of casein and which appeared to be required for the production of tetanus toxin. The addition of asparagine gave results of doubtful significance.

Binkley and Olson (38) stated that glutamine was required for the hydrolysis of glutathione by enzymes present in kidney tissues. They noted that glutamine was not hydrolyzed by the reaction and concluded that it must act as a coenzyme. Penicillin and bromosulfalein inhibited the hydrolysis of glutathione completely. Archibald (4) has shown that bromosulfalein is a potent inhibitor for glutaminase. This suggests that a common enzyme might be involved in both reactions. These authors suggest the following as possible steps in the hydrolysis of glutathione to cysteinylglycine:

(a) Glutamine + enzyme = enzyme-amide + glutamic acid

(b) Enzyme-amide + glutathione = glutamine + cysteinylglycine + enzyme.

Mardashev and Semina (39) incubated rat liver slices and glyoxalic acid with asparagine, aspartic acid, NH_4Cl, glutamine and glutamic acid and demonstrated the synthesis of glycine. Glutamine was most effective followed by asparagine, glutamic acid, and aspartic acid. They postulated transamination as a possible course and suggested the

participation of 2 enzymes causing condensation of the amide group with the carbonyl group of the glyoxalic acid followed by reduction and hydrolysis into glycine and amino dicarboxylic acid. Meister and Tice (40) observed that deamination of glutamine by rat liver extracts and purified preparations of rat liver was accelerated by a number of α-keto acids. Acids possessing β-, γ-, or δ-keto groups were ineffective along with oxalacetate, α-ketoisovalerate, α-keto-β-methylvalerate, and pyruvamide. They explained their results on the basis of a two-step reaction involving an α-keto acid-catalyzed deamidation of glutamine giving ammonia and glutamic acid, followed by a transamination reaction between the α-keto acid and glutamate. With glutamine labeled in the amide position with N^{15}, all the isotopic nitrogen was recovered as ammonia after incubation with pyruvate or phenyl pyruvate, demonstrating unequivocally that the ammonia formed arises from the amide group of glutamine.

Of particular interest at this laboratory are experiments concerned with the interrelationships of glutamine to protein metabolism. The work of Tigerman and MacVicar (30) has suggested that factors which cause a rise in the ammonia production within an animal (rats) produce a concomitant increase in glutamine level of body tissue. They also demonstrated (41) that the oral administration of glutamine and ammonium carbonate caused an increase in tissue glutamine content, while glutamic acid produced no effect. The simultaneous feeding of ammonium carbonate and glutamic acid produced significant increases in tissue glutamine levels. They concurred with the hypothesis that glutamine might serve as a mechanism for storing ammonia. Other factors known to cause an increase in ammonia production, i.e., thyroid-active substances, and cortical hormones, were subsequently investigated for

the purpose of further elucidating the role of amides in protein metabolism. Tigerman (3) reported a substantial increase in tissue glutamine level in rats which were rendered hyperthyroidic by feeding a ration containing an iodinated casein preparation. These findings were largely substantiated by later investigations by this investigator. The administration of cortisone, however, which also increased nitrogen excretion, was shown to have no effect upon tissue glutamine. This suggested a possible difference in the effect of the two hormones upon nitrogen metabolism. Albright (42) suggested that the 11-oxygenated steroids were concerned with an antianabolic, rather than a catabolic effect on proteins. Opposed to this theory are the investigations of Hoberman (43) and White and Dougherty (44). Hoberman, in experiments with N^{15}-labeled glycine claimed that in 2 days, fasting rats lost up to 8% of their carcass protein when injected with cortisone. His calculations gave evidence for a protein catabolic effect for cortisone. White and Dougherty hypothesized a catabolic effect upon carcass protein by thyroid hormone and a similar effect on the protein of lymphoid tissue for adrenal hormones. They think that the adrenals might have some effect on carcass proteins mitigated through an influence of the cortical secretions on the thyroid gland. They suggest a synergistic effect for the two hormones. Clark (45) conducted an experiment employing N^{15}-labeled glycine which was fed to adrenalectomized rats which received cortisone, adrenalectomized rats, sham operated rats which received cortisone, and sham operated controls. By using the method of Sprinson and Rittenberg (46) they calculated the rate of protein synthesis and demonstrated that cortisone administration resulted in a significant decrease in the rate of protein synthesis. These findings support the theory of Albright.

Roderuck (47) analyzed for glutamine, skeletal muscle of hamsters, rabbits, and guinea pigs, in which experimental dystrophy had been induced by vitamin E deficiency. They found that glutamine level was reduced in guinea pigs; less so in rabbits. Roderuck and coworkers (48) in another paper reported a reduction to 70% of the level in controls, of glutamine in skeletal muscles of dystrophic rabbits and guinea pigs.

Bartlett and coworkers (49, 50, 51, 52) conducted a number of studies on the mechanism of nitrogen storage in which they studied the effects of anterior pituitary growth hormone preparations on plasma and muscle glutamine, free amino acids, and kidney glutaminase and muscle transaminase enzymes. They (49) described two phases of hormonal action in their experiments with dogs, an anabolic phase characterized by low nitrogen excretion followed by a catabolic phase which was accompanied by an increase in nitrogen excretion. Plasma glutamine levels rose during the anabolic phase and fell during the catabolic phase, which is not in agreement with the hypothesis that protein catabolism enhances glutamine synthesis. They concluded that changes in plasma glutamine nitrogen did not account for the observed changes in plasma amino acids, or for the nitrogen which was stored following growth hormone administration. In experiments on kidney glutaminase (50), they observed that large doses of hormone administered to four-week-old hypophysectomized rats caused an increase in kidney glutaminase whereas doses just large enough to produce body weight gain had no effect on the enzyme. They also concluded that the capacity of the normal rat liver to deamidate glutamine in the presence of pyruvate was unaffected by the administeration of growth hormone (51). In the studies of muscle transaminase (52) their results confirmed the concept that growth hormone may produce some effect through diminished amino acid catabolism

as the hormone caused a significant decrease in muscle transaminase of immature hypophysectomized rats. They demonstrated that concentrations of glutamine carboxyl N in skeletal muscle are lower in hypophysectomized rats receiving growth hormone and in rapidly growing normal rats than in rats which have had their growth arrested by hypophysectomy. These observations are not in agreement with our findings in an experiment using normal animals in an induced state of growth. This may not be of importance, however as Bartlett and Glynn (52) were comparing rapidly growing animals with hypophysectomized animals on a growth plateau. No data were given for normal animals fed ad libitum vs. normal animals in an enhanced state of growth.

III. Tissue Glutamine Levels.

Dawson and Williams (24) quote values for normal rat liver glutamine as 101 mg.%, hypoglycemic, 55 mg.%. Richter and Dawson (53) give the normal glutamine content of rat brain as 79 mg.%. Mardashev and Mamaeva (6) give the value for rabbit liver and kidney as 52.1 and 21.7 mg.% respectively. Astrup and Munkvad (7), Örström and Örström (29) give values for human plasma glutamine of 6.19-9.83 and 10 mg.% respectively.

I. METHODOLOGY

Care of Animals

At the beginning of each experiment the animals were selected at random and placed in individual cages, except where paired experiments were being conducted. In these cases the animals were paired on the basis of weight and litter and members of each pair were assigned to treatment at random. Unless otherwise stated the following ration was used as a standard basal ration: Casein, 20%; sucrose, 71; corn oil, 5; salt mix (Hegstead *et al.*, *J. Biol. Chem.*, 138, 460 (1941).) 4. Each kilogram of ration was supplemented with the following vitamins: thiamine, 4 mg.; riboflavin, 5; pyridoxine, 3; calcium pantothenate, 20; nicotinic acid, 20; folic acid, 1; inositol, 20; p-aminobenzoic acid, 20; choline chloride, 1 gm. In addition, vitamins A and D were administered every 3rd day by dropper. The animals were fed *ad libitum* for an equilibration period of 8 to 10 days prior to beginning treatment. The animals received fresh water daily.

Removal of Tissues

At the termination of the experiment the animals were killed by exsanguination under light ether anesthesia. The tissues were rapidly removed and immediately frozen and stored at -15°C. until analyzed. Analysis was performed as soon as practicable after termination of the experiment, to avoid undue decomposition of glutamine.

Determination of Glutamine

In the earlier investigations in this laboratory, the enzymic

method of Archibald (4) was used for the determination of glutamine. The author found, however, that there were several serious drawbacks to this procedure when applied to tissues. It was difficult to obtain and preserve active enzyme preparations, and, as Archibald pointed out, the method was not satisfactorily applicable to tissues without special precautions as ammonia was liberated from adenosine containing compounds by the enzyme preparation.

The acid hydrolysis method suggested by Pucher, Vickery, and Leavenworth (54) was ultimately found most satisfactory after considerable modification of those tested despite its lack of specificity. A weighed sample of tissue (approx. 1 to 2 gm.) was homogenized in enough water to make a total volume of 29 ml. One ml. of 6 N H_2SO_4 was then added to liberate any "bound" glutamine and the homogenate was centrifuged for 5 min. at 1500 R.P.M. A 10 ml. aliquot was added to 5 ml. of 2 1/2% sodium tungstate (there being enough acid added in the homogenization procedure to precipitate the protein with the tungstate) and again centrifuged as above. Two 5 ml. aliquots were taken from the clear supernatant liquid for analysis. To one aliquot was added 1 ml. of 6 N H_2SO_4 and digested for exactly 10 min. in a boiling water bath to hydrolyze the glutamine. The other aliquot was buffered to about pH 8-9 with a phosphate-borate-NaOH buffer and distilled with aeration for 20 min. to determine preformed ammonia. The hydrolyzed sample was then treated in a similar manner after adding enough NaOH solution to neutralize the H_2SO_4, and the glutamine was calculated as the difference in ammonia level of the two aliquots. Ammonia was determined by nesslerization of the distillate which was trapped in a measured quantity of dilute H_2SO_4. In some of the earlier experiments, glutamine was

determined as above except aliquots of the tungstate-protein filtrate were nesslerized directly before and after hydrolysis. It was noted, however, that occassionally a turbidity would develop during nesslerization which would give erroneous results when optical transmittance was measured. With some tissues, abnormally high results were consistently obtained the cause for which is not known. Because of these difficulties the distillation method was adopted, even though it was more time consuming.

II. EFFECT OF HORMONES ON TISSUE GLUTAMINE LEVEL

Effect of Thyrotoxicosis on Tissue Glutamine Level

Tigerman (3), on the basis of 2 trials, reported significant increase in glutamine levels in the liver, kidney, heart, spleen, muscle and brain tissues of animals on a sodium proteinate ration supplemented with iodinated casein (Protamone). Thyroxine is known to enhance protein catabolism, and since tissue glutamine levels correlated with decreased growth rate, he advanced the theory that factors tending to increase the breakdown of body protein result in increased accumulation of glutamine in tissues. He also pointed out that the administration of thyroxine had an effect upon the rate of release of adrenal cortical hormones, which in turn play an important role in the mobilization of nitrogen from tissues. He suggested that this influence on adrenal cortical hormones may also be a direct factor in enhancing the level of tissue glutamine and the thyroid activity a secondary one.

In another research project being conducted in this laboratory, a study was being made of the effect of administration of various materials upon the growth-depressent effect of thyroid toxicity (55). Advantage was taken of the availability of tissues from these animals to measure the glutamine level in order to determine if these anti-thyroid materials would register any effect.

Experimental. Weanling rats (Sprague-Dawley strain) were put on a basal ration for 10 days. The ration consisted of the following: soybean proteinate preparation (sodium proteinate, Archer-Daniels-Midland Company, Minneapolis, Minn.) to provide 20% protein; salts

(Hegstead), 4; cottonseed oil, 5: and cornstarch to make 100%. Vitamins were added at twice the level given above in order to be certain of an adequate supply. For the ensuing 2 weeks the ration was modified to contain 0.25% iodinated casein (Protamone, Cerophyl Laboratories, Kansas City, Mo.). One group of animals was given this ration; another group was supplemented with 2% 1:20 liver powder; and a third group was fed 25 micrograms of vitamin B_{12} per kilo in addition. The glutamine levels found in tissues of these animals at the end of this period are shown in Table I.

Table I

Tissue Glutamine Levels From Rats on Soybean Proteinate Rations Supplemented with Iodinated Casein, Liver Extract and Vitamin B_{12}.

Supplement	Iodinated Casein	1:20 liver powder	Vitamin B_{12}	"F" Value
	Glutamine concentration (mg.%)*			
Tissues				
Liver	117	126	69	10.71
Kidney	135	111	139	.95
Heart	320	186	157	6.83
Brain	214	119	196	7.02
Spleen	148	128	88	2.06
Avg. gain in wt./ day for 2 weeks	1.26gm.	2.27gm.	1.84gm.	
No. of animals	10	9	10	

*These values obtained by acid hydrolysis and direct nesslerization.

In agreement with Tigerman's findings, thyrotoxicosis resulted in elevation of tissue glutamine levels in all tissues. It will be noted that vitamin B_{12} supplementation resulted in a decrease in glutamine level of tissues except kidney, and liver supplementation resulted in a decrease in glutamine level in all tissues except liver. These data were subjected to statistical treatment by analysis of variance (Snedecor). Values of "F" showed that the difference between treatments was highly significant

(>0.01) for all tissues except kidney and spleen. In the latter organ, the "F" value approached significance at the 5% level. Whether this effect is a specific effect on glutamine metabolism or whether the observed changes are secondary to an improved growth rate is not known. All animals continued to show symptoms of severe hyperthyroidism (hyperpnea, tachycardia, nervousness).

Effect of Adrenal-Cortical Excess on Tissue Glutamine Levels

As was mentioned previously, the administration of thyroxine has a marked effect on the rate of release of the hormones of the adrenal cortex. As the adrenal cortex has also been associated with the metabolism of amino acids (and the mobilization of tissue proteins for catabolism), it was thought that at least part of the effect of thyrotoxicosis on tissue glutamine content might be a result of enhanced cortical activity. It was deemed advisable to determine the effect of cortisone on amide nitrogen metabolism.

Experimental. Several trials of this experiment were made. In the first trial adult male rats were fed the standard basal ration ad libitum for a 10 day equilibration period and during the experiment. At the end of the equilibration period the rats were divided into two groups: each animal of one group receiving an initial injection of 25 mg. of cortisone acetate intraperitoneally, followed by three daily injections of 10 mg. each; the second group received similar sham injections of normal saline solution. The rats were killed 24 hours following the last injection. A daily weight record was maintained. From the beginning of cortisone treatment until the rats were sacrificed the following average weight changes occurred: Control, -0.1 gm.; cortisone-treated rats, -31.1 gm. It appears almost certain that

the experimental animals were in negative nitrogen balance on the basis of weight losses. The glutamine level of various tissues was determined and these data are recorded in Table II.

Table II

Tissue Glutamine Levels in Rats Following Intraperitoneal Injections of Cortisone

	-Cortisone Injected			Control		
Trial No.	1*	2*	3**	1*	2*	3**
	Glutamine concentration (mg.%)					
Tissues						
Liver	92	94	97	115	117	102
Kidney	73	92	79	65	98	82
Heart	178	---	114	196	---	120
Brain	147	178	71	141	162	71
Muscle	27	68	---	56	66	---
Spleen	---	64	95	---	79	97
Lung	---	50	---	---	54	---
No. of animals	9	9	13	8	9	13

* These values obtained by direct nesslerization.
** These values obtained by distillation and nesslerization.

To confirm the results of trial 1, two additional trials were conducted, the results of which are also shown in Table II. In the first trial a marked anorexia was noted among the animals receiving cortisone injections. To eliminate any possible effect from difference in food consumption, a paired feeding technique was used in trials 2 and 3. The food intake of the control animal of each pair was limited to the total amount of ration consumed by the cortisone-injected rat during the preceeding 24-hour period. In trial 2 the rats received cortisone at the same level on a body weight basis as those in trial 1 but the experiment was extended for two additional days. In trial 3 the injections were as in trial 2 except that the dose was increased by 1/2 on the last two days. The experimental animals lost weight in both trials while the controls did

not. It was noted that the differential weight loss was greatest at about 36 to 48 hours and then decreased with little or no loss beyond the 5th day. Since it was thought that there might be an effect on the tissue glutamine content at the peak of weight loss, as previous experiments showed that conditions which induced negative nitrogen balance resulted in increases in tissue glutamine level, another trial was made in which the injected animals received two injections of 25 mg. of cortisone 24 hours apart and were sacrificed 40 hours after the initial injection. During this period the injected group lost an average of 10.8 gms. while the control group gained 6.6 gms. The results of the tissue analysis for control and treated animals, respectively, were as follows: Liver, 118 mg.% and 129; kidney, 52, 50; heart, 94, 87; brain, 80, 84; muscle, 71, 83; and spleen, 53, 60. These analyses were obtained by acid hydrolysis and distillation. The number of animals in the groups were 12 and 13, respectively. These animals were not pair fed as the experiment was of such short duration that pair feeding would have offered no advantage.

Effect of Growth Hormone Preparations on Tissue Glutamine Levels

In view of the effect on tissue glutamine content of factors which stimulate protein catabolism it appeared desirable to investigate the effect of a factor which is known to enhance nitrogen retention and elevate the rate of protein synthesis, i.e., the growth hormone of the anterior pituitary. Bartlett and Glynn (52) reported a decrease in skeletal muscle glutamine level in rapidly growing normal rats and hypophysectomized rats receiving growth hormone as compared to the level of glutamine in the muscle of rats whose growth had been arrested by hypophysectomy.

Experimental. Twenty young female rats weighing about 110 to 130 gm. were assigned at random to two groups of equal size after a preliminary equilibration period of 10 days on the standard basal ration previously described. They were placed in metabolism cages and fed 15 gm. of the basal ration daily. The feed refused was collected and weighed each day. Urine was collected in dil. H_2SO_4 to avoid loss of ammonia. Total nitrogen was determined daily on urine and feces by the Kjeldahl method.

The group assigned to hormone treatment (designated 11-E through 20-E) were injected with 1 ml., containing the equivalent of 2 mg. of growth hormone (Armour's standard) daily for 3 days. At the end of this 3 day period it was noted that the animals were suffering from anorexia as they were consuming considerably less of the ration than the controls. It was thought that perhaps the hormone was being administered in too large dosage, so for the remainder of the experiment the dosage was reduced to 1 mg. daily. Almost immediately the effect was noted in N balance studies. The animals consumed as much or more than the controls, as shown by Table III. The control group of animals received injections of normal saline daily.

It will be observed from the data in Table III that the animals which were injected with the growth hormone preparation gained considerably more weight during the last 4 days of treatment. Their food consumption was increased over the controls. The amounts of excreted nitrogen by the two groups are not strikingly different but the difference in amount of nitrogen retained is highly significant (> 0.01). That the animals were in a state of elevated nitrogen retention is shown by the proportion of the consumed nitrogen which was retained as shown by the right-hand column in the table.

Table III

Nitrogen Metabolism of Rats Receiving Growth Hormone

Rat No.	Initial* wt.,gm.	Final Wt.,gm.	Wt. Change	N consumed mg.**	N excreted mg.**	N balance	N retained / N consumed
1-C	135	142	+ 7	1035	630	+405	.39
2-C	135	154	+19	1205	721	+484	.40
3-C	132	140	+ 8	1070	564	+506	.47
4-C	138	148	+10	1023	571	+452	.44
5-C	132	145	+13	1074	607	+467	.43
6-C	135	139	+ 4	751	489	+262	.35
7-C	139	148	+ 9	996	702	+294	.30
8-C	141	154	+13	1187	730	+457	.39
9-C	123	146	+23	1283	676	+607	.47
10-C	122	138	+16	936	482	+454	.49
11-E	122	150	+28	1320	509	+811	.61
12-E	127	157	+30	1411	514	+897	.64
13-E	122	146	+24	1296	520	+676	.52
14-E	116	143	+27	1355	639	+716	.53
15-E	156	174	+18	1264	622	+642	.51
16-E	139	158	+19	1202	600	+602	.50
17-E	118	142	+24	1430	635	+795	.56
18-E	134	154	+20	1359	736	+623	.46
19-E	130	156	+26	1478	682	+796	.54
20-E	120	149	+29	1485	621	+864	.58

* Commencing with the reduced hormone dosage (4th day of treatment)
**For 4 days.

While the animals were still in a state of elevated nitrogen retention, the experiment was terminated. The animals were sacrificed and tissues obtained in the manner already described. The results of the tissue analysis for glutamine are given in Table IV.

The small differences noted are not believed to be significant. From the results obtained it appears that an induced state of rapid growth in normal animals does not result in a decrease in the tissue glutamine content of the tissues studied, when compared to normal animals fed ad libitum on a diet containing adequate amounts of the constituents required for normal growth. These findings are not necessarily believed to be in disagreement with the reported findings of Bartlett and Glynn (52), since

Table IV

Effect of Growth Hormone Administration on Tissue Glutamine Levels in Rats

Tissue	Glutamine (mg.%)* Control	Growth Hormone Injected
Liver	88	86
Kidney	38	43
Brain	45	51
Heart	49	45
Spleen	44	44
Muscle	64	62
Number of Animals	10	10

* Values obtained by distillation and nesslerization.

they made no comparison of glutamine levels of animals in normal growth with animals in enhanced growth. They did find that arresting growth by hypophysectomy resulted in an increase in tissue glutamine content, however.

Discussion

An induced state of thyrotoxicosis has been shown to result in an increase in tissue glutamine content in rats (3), whereas no effect was observed in tissue glutamine during negative nitrogen balance induced by massive doses of cortisone or during the nitrogen retention elicited by administration of growth hormone preparations. These observations would seem to preclude the assumption that all factors which bring about a response in nitrogen excretion or retention result in a concomitant effect upon glutamine synthesis. An obvious conclusion is that thyroid-active substances have a direct effect upon glutamine synthesis. A further inference is that the mode of intervention in amino acid anabolism and catabolism of hormones of the thyroid and adrenal glands may be different. It is also evident that the growth hormone of the anterior pituitary gland should not be merely relegated to a simple opposing or counteracting

action in its role in nitrogen retention and growth promotion.

Furthermore, these findings indicate that the role of glutamine in metabolic processes is a complex one and not merely solely a means at the disposal of the organism by which it can retain nitrogen in the body in a non-toxic form.

III. THE EFFECT OF CORTISONE ON PROTEIN SYNTHESIS

Both thyroxine and cortisone excess induce depressed growth or loss in weight and negative nitrogen balance. Glutamine levels in the tissue were significantly increased in thyrotoxicosis and were unchanged in the face of massive doses of cortisone. It appeared, therefore, that the mode of action of the two hormones upon protein metabolism must be different because of the varying effect on glutamine level. Clark (45) presented evidence in support of the concept that the effect of cortisone on protein metabolism in an "antianabolic" rather than a "catabolic" effect by measuring the rate of protein synthesis under various treatments. Recent work by Engle (56) ascribed a permissive role to the adrenal secretions in response of the organism to stress. He concluded that it was stress that elicited nitrogen excretion, but presented evidence to show that adrenal cortical secretions were required. They lower the threshold to the stress response. He also demonstrated the involvement of nutritional status, as this excess nitrogen excretion could largely be prevented when caloric intake was increased.

In an effort to determine the nature of the effect of the cortical steroids upon nitrogen metabolism, an experiment was conducted in which N^{15} labeled protein was fed to rats receiving cortisone and to control animals. It was hoped that by measuring the amount of N^{15} retained and excreted some light could be shed upon the way in which cortisone mediated in protein synthesis and breakdown.

Experimental

The problem of obtaining labeled protein material that would be readily metabolized by the animals was solved by growing brewers yeast

(Saccharomyces cerevisiae) in a culture media containing $N^{15}H_4Cl$ as a source of nitrogen. The culture media had the following composition: dextrose, 210 gm.; KH_2PO_4, 6; $N^{15}H_4Cl$ (32 atom percent excess), 4.16; $MgSO_4$, 1.2; $FeSO_4$, 0.06; $MnSO_4$, 0.24; and 3 mg. of each of inositol, thiamine, biotin, pantothenic acid, pyridoxine, and nicotinic acid. The 4.16 gm. $N^{15}H_4Cl$ (calculated amount) was obtained from converting 6.1 gm. $N^{15}H_4NO_3$ to $N^{15}H_3$ and absorbing the $N^{15}H_3$ in HCl solution. The excess HCl was neutralized with NaOH. The pH of the media was adjusted to 4.3-4.5 the media diluted to 3 liters and autoclaved. This was innoculated with a yeast cake which had been suspended in 400 cc. of sterile water. The temperature was maintained at 33°C. by means of a water bath which was thermostatically controlled. The media was constantly aerated by using an aspirator pump to pull air through the system. The air was dispersed into fine bubbles by means of a sintered glass dispersion disk. Sterile cotton plugs were used to prevent contamination. At the end of 36 hours the cells were thrown down by centrifugation and washed twice with distilled water. The yeast was dried in an oven at 75°C. and ground in a Wiley mill. The yield was 17.8 gm. dry yeast, calculated to contain about 8 gm. of protein (a parallel control run produced yeast which contained 47.5% protein by Kjeldahl analysis). Isotopic analysis showed that the nitrogen from this yeast contained 24.53% N^{15}.

The basal ration was made up as follows: dried yeast (compressed yeast)obtained from a local bakery 250 gm.; vitamin test casein, 28; cornoil, 50; salt mix (Hegstead), 40; and cerelose (dextrose), 632. This was supplemented with vitamins at the same level as described for the standard basal ration used previously. This ration contained about 16% protein.

Ten rats were paired (litter mates) and assigned to treatments at

random. They were put into individual cages and fed the above described ration ad libitum, for an equilibration period of 6 days. These animals were then placed in metabolism cages and fed 10 gm. the ration daily. Urine and feces collections were made at 24 hour intervals and total nitrogen determined by Kjeldahl method. Refused feed was weighed daily. On the 3rd day in the metabolism cages (after a 48 hour adjustment period) the treatment group received 25 mg. cortisone via intraperitoneal injection. They were given 12.5 mg. daily thereafter until the experiment was terminated. The control group received parallel sham injections of normal saline. At 48 hours after the cortisone treatment was begun all animals were fed 7 gm. of ration which contained the isotopically labeled protein. (The amount of the ration was cut down to 7 gm. so that all animals would consume all the ration and thus receive the same amount of labeled protein. This ration was fed at the peak of the effect of the cortisone based on previous experience.) The labeled ration was made in the same proportions as the basal ration, but analysis showed that it contained a slightly lower amount of nitrogen. Isotopic analysis showed it contained 19.85% N^{15}. The day following this the animals were again fed the usual 10 gm. of basal ration. The animals were sacrificed 48 hours following the feeding of the labeled ration and liver and muscle tissues were removed for further experimentation. The urine and feces collected these two days were analyzed for N^{15} content as well as total nitrogen. Control animal No. 5 contracted a respiratory disease and was discarded.

Results and Discussion

Table V shows the effect of cortisone on nitrogen balance. It will be observed that all the control animals were in positive balance during the four days of treatment, while all five of the animals which received cortisone were in negative nitrogen balance for this same period. The

weight changes of each individual animal are also given in the table. As the quantity of N consumed during this period was greater for the treated group than for the controls (Table V) the loss of weight and negative nitrogen balance must be attributed to the effect of the cortisone. It will also be noted that the values for percent of injected N^{15} which was excreted in the urine during the 48 hours following administration of the labeled protein for each individual rat are given. The average for the control group is 24.55%, and for the treated group is 45.66%, which is significant at the 1% level as shown by "t" test (Snedecor). The values of protein synthesis were calculated by the method of Sprinson and Rittenberg (46). Clark (45) reported values for rates of protein synthesis from N^{15} labeled glycine in normal and cortisone treated rats, which agree reasonably well with these values. Clark's values were, for normal rats administered with cortisone, 1.10 gm./kilo (one animal), and for normal controls, 1.59 gm./kilo (average of 2 animals). The corresponding averages from Table V are 1.35 and 1.85 respectively. The difference in these values could easily be explained by the assumption that the labeled amino acids in the protein from the yeast would be more readily utilized for the synthesis of protein than isotopically labeled glycine. This same explanation could be used to justify the slight differences in the percentages of N^{15} excreted during 48 hours. Clark reported 57% (one animal) and 37.5% (average of 2 animals) for normal rats receiving cortisone and normal controls, respectively, whereas the averages of the values given in Table V are somewhat lower.

Table VI shows clearly the hypertrophy of liver tissues which results from cortisone treatment, the average weight of livers from the control group being 5.72 gm. as compared to 7.64 gm. for the cortisone treated animals. It will also be noted that there appears to be a lower protein

Table V

Data on Nitrogen Metabolism and Protein Synthesis

Rat No.	Control				Cortisone Treated				
	1C	2C	3C	4C	1T	2T	3T	4T	5T
Initial wt., gm.	158	156	152	160	164	166	152	162	148
Final wt., gm.	166	158	144	156	150	147	140	138	136
Change in wt. gm. in 4 days	+8	+2	-8	-4	-14	-19	-12	-24	-12
N consumed, mg.	835	913	742	912	914	914	913	914	914
N excreted, mg. in urine + feces	677	785	682	741	1004	1018	947	1268	1000
N balance	+158	+128	+ 60	+171	- 90	-104	- 34	-354	- 86
$\%N^{15}$ excreted in urine, 48 hours	24.4	26.5	25.0	22.3	40.9	41.7	42.5	55.0	48.2
S_K*	1.78	1.80	1.75	2.06	1.17	1.65	1.21	1.38	1.34

*Rate of protein synthesis, gm. N/kilo/day

content in the livers from the cortisone group judged from the Kjeldahl analyses. This observation is supported by the findings of Germuth et al. (61) who reported that in animals treated with compound E there were extensive deposition of glycogen and fat in the liver. They also found that compound E produced atrophy of the lymphoid tissues, including the thymus and spleen, a lymphocytopenia, and a lipemia and focal necrosis of skeletal muscle. The total N^{15} content of the cortisone group is somewhat higher than that of the control group, which also shows an increased rate of protein synthesis in liver tissue. These data further support the findings of Clark (45). The concentration of isotopic N in muscle tissue (gastrocnemius) is lower in the animals which received cortisone than in the control group suggesting a diminished rate of protein synthesis in this tissue.

On the basis of nitrogen balance studies, rate of protein synthesis,

Table VI

Composition of Liver and Muscle Tissues

Rat No.	Liver				Muscle
Rat No.	weight	%protein	$\%N^{15}$	total mg. N^{15}	$\%N^{15}$
1C*	5.26 gm.	19.88	----	----	----
2C	5.81	19.67	1.17	2.14	0.61
3C	5.86	18.09	1.28	2.17	0.60
4C	5.96	19.10	1.11	2.13	0.71
avg.	5.72		avg.	2.147	
1T	8.83	16.62	1.22	2.86	0.49
2T	7.49	17.27	1.00	2.07	0.51
3T	7.98	18.39	1.28	3.00	0.51
4T	7.76	17.03	1.11	2.35	0.51
5T	6.14	18.80	1.29	2.37	0.51
avg.	7.64		avg.	2.53	

* Samples for isotope analysis contained air.

and incorporation of N^{15} in muscle tissue, it appears evident that the effect of cortisone is to inhibit the synthesis of protein rather than enhance the mobilization of existing tissues. The effect on liver is the opposite to this as the liver from the treated animals contained more protein and N^{15} and were larger than those from the controls.

IV. HISTIDINE ADMINISTRATION AND GLUTAMINE CONTENT OF RAT TISSUES

Histidine is recognized as one of the amino acids essential for growth of the rat, but the disposition of that in excess of the growth requirement is uncertain. Edlbacher and Neber (57) suggested that L-histidine is converted to glutamic acid during the process of metabolism. Crookshank and Berg (58) concluded that L-histidine was probably not converted to other derivatives of imidazole but that the ring was ruptured with the formation of an alpha-amino compound. Featherstone and Berg (59) in studies of the metabolism of histidine by kidney and liver slices suggested that L-histidine might be converted to alpha-amino products other than glutamic acid. Crookshank and Clowdus (60) studied the blood levels of glutamic acid, histidine, total imidazole, and amino nitrogen before and after feeding single doses of D- and L-histidine to rats. They concluded that either no conversion to glutamic acid occurred, the conversion proceeded too slowly to be detected by their methods, or that any glutamic acid formed was rapidly converted to some other metabolite retaining the alpha-amino group. In line with this latter postulation, it seemed possible that if glutamic acid were formed it might be converted to glutamine by combination with ammonia. Tigerman and MacVicar (41) had demonstrated a rise in tissue glutamine levels in rats resulting from the simultaneous administration of ammonium ion and glutamic acid. This could account for the fact that no increase in blood glutamic acid was observed by Crookshank and Clowdus following histidine ingestion. The effect of histidine administration upon the glutamine level in certain rat tissues was therefore investigated.

Experimental

Mature rats were injected intraperitoneally with L-histidine at the

level of 0.16 gm. of amino nitrogen per kg. body weight. The solution of histidine for injection was prepared by dissolving histidine monohydrochloride in normal saline and adjusting the pH to 7.4 by addition of sodium bicarbonate. To determine the effect of the NaCl and NaCl with $NaHCO_3$, control groups were simultaneously injected with NaCl and with NaCl + $NaHCO_3$ at the same level which was used with histidine injection. The rats were killed 15 minutes after injection and tissues removed for analysis. In previous work, this period has been shown to be adequate for mobilization of the injected material from site of injection. The results are shown in Table VII.

Table VII

Effect of Histidine Injection on Glutamine Level in Rat Tissue

Treatment Number*	1	2	3
	Glutamine (mg. % ± std. dev.)**		
Tissues			
Liver	156 ± 39	114 ± 32	96 ± 36
Kidney	50 ± 18	68 ± 20	67 ± 15
Heart	172 ± 22	166 ± 28	187 ± 22
Number of animals	5	5	5

*1 - 0.9% NaCl injected
2 - NaCl + $NaHCO_3$ injected
3 - NaCl + $NaHCO_3$ + histidine injected
** These values obtained by direct nesslerization.

Results and Discussion

Comparison of the values found for the animals receiving treatments one and two shows that the administration of histidine did not induce an increase in the level of glutamine in the tissues examined. Thus, glutamic acid, if formed from histidine, is apparently not converted to glutamine to any appreciable extent. No support for the hypothesis that glutamine is the alpha-amino compound reported as a result of histidine

metabolism is afforded by these results.

It will be noted that the liver glutamine level of the animals receiving bicarbonate is reduced with respect to the animals receiving only saline. It is not believed that any significance should be attached to this observation, since the usual value for liver glutamine is near 100 mg.%. Both previous and subsequent tests have failed to reveal any influence of the injection of normal saline, and the observed differences are therefore attributed to the rather wide variation between animals observed and the small number of animals involved.

GENERAL DISCUSSION

As was emphasized in the literature review, glutamine has been associated with a large number of important metabolic reactions in both animals and plants. Glutamine seems to have a universal distribution in animal tissues. It is becoming increasingly evident, as additional investigations are reported, that glutamine occupies an important position in the nitrogen metabolism of most forms of life. It may well be that the energy which is associated with the amide bond is an important factor in the function of glutamine. In fact, it does not seem too unreasonable to postulate a "high-energy" nitrogen bond similar to the well established "high-energy" phosphate bond and the more recently postulated "high-energy" sulfur bond. By virtue of this high-energy content glutamine could serve as a source of amino groups for many of the reactions involving nitrogen transfer, being regenerated from ammonia and glutamic acid at the expense of energy from adenosine triphosphate or some other high-energy phosphate compound. An example of such a situation has only recently come to the attention of the author. Weil-Malherbe (62) found that the addition of glutamine and either inosine triphosphate or a mixture of inosinic acid and creatine phosphate to dialyzed brain homogenates, resulted in an NH_3 formation in excess of that of control experiments. He interpreted these results as indicating the transfer of the amide group of glutamine to inosine triphosphate or another inosine phosphate, possibly at the expense of high-energy phosphate. Kalckar and Rittenberg (63) had suggested amides as a possible source of nitrogen for the rapid rejuvenation of muscle adenylic acid, as they observed a high concentration of N^{15} in the 6-amino position of adenylic acid following administration of labeled ammonium citrate.

Muscle glutamic acid had only 1/5 as much isotope as the adenylic acid while the isotope concentration was higher in amide nitrogen.

The results of the investigations reported in this thesis cannot be considered as contributing to this hypothesis directly. They do serve, however, to emphasize that the function of glutamine is more complex than providing a means available to the organism of detoxifying ammonia or storing and retaining amino nitrogen. It was shown that thyrotoxicosis resulted in an increase in tissue glutamine. As this state is also accompanied by increased nitrogen excretion, it might be assumed that the increase in glutamine was a direct result of the increased rate of nitrogen release. Cortisone, which also brings about an increased rate of release of nitrogen, did not cause a similar increase in tissue-glutamine level, however. Growth hormone preparation from the anterior pituitary gland, which enhances nitrogen retention, did not result in a concomitant decrease in tissue glutamine. From these observations it might be suggested that nitrogen retention or loss per se does not have a direct effect on glutamine metabolism. In those conditions in which an increased rate of mobilization of the body protein reserves occurred (starvation, amino acid depletion, thyrotoxicosis) glutamine was found to be increased. If cortisone has an anti-anabolic effect rather than a pro-catabolic one, the differences observed in glutamine level might be rationalized. These results could also be explained by assuming that thyroid-active substances exert a direct stimulating effect on glutamine synthesis.

That glutamine is functioning in some more organic role than as a storage form for NH_4^+ is indicated by the fact that increasing the level of ammonia in the blood did not result in a material increase in tissue-glutamine levels. This finding is further supported by the findings

reported in Part II of this thesis. Here it was shown that the intraperitoneal administration of ammonium ion as the citrate and glutamate salts did not result in glutamine levels in liver and kidney in the rat which were out of the range considered to be normal for this animal.

The action of cortisone on amino acid metabolism was shown to be of an anti-anabolic rather than a catabolic effect. This was accomplished by feeding of protein materials labeled with N^{15} and calculating the rate of protein synthesis in normal untreated rats and in normal cortisone-injected rats. Nitrogen metabolism studies on these animals demonstrated that administration of this hormone resulted in a marked increase in nitrogen excretion. Increased nitrogen retention induced by growth hormone should be studied in a similar manner to determine the site of action on amino acid and protein metabolism.

These investigations have further emphasized the complexity of nitrogen metabolism. It is evident that the ultimate role of amides in nitrogen metabolism will be determined only by continuing and extending studies of the type reported here. The importance of this fraction, especially in the brain and nervous tissue, as a functional part of intermediary metabolism of amino acids and ammonia fully justifies an accelerated research program.

SUMMARY

It was demonstrated that vitamin B_{12} and a liver preparation, which checked the growth-retarding action of thyroid-active substances in the rat, resulted in a reduction toward normal of the glutamine content of most tissues analyzed. This may have been an indirect result of improved growth or a direct opposing action of the vitamin to the effect of thyroid-active substances on glutamine synthesis.

Cortisone, though it greatly increases nitrogen excretion, had no effect on tissue glutamine level. Likewise, a growth hormone preparation, which was shown to greatly increase nitrogen retention, did not result in a decrease in tissue glutamine content.

Experiments, utilizing protein labeled with N^{15}, lent support to the theory that cortisone exerts an anti-anabolic rather than a catabolic effect on tissue protein. Nitrogen balance studies showed that massive doses of cortisone resulted in negative nitrogen balance in rats. It also decreased the rate of protein synthesis in most tissues. The rate of protein synthesis in liver, however, was increased as was shown by the larger content of isotope and by the hypertrophy of the organ. These experiments suggest a difference in the mode of action of thyroid active substances and adrenal cortical hormones on protein metabolism.

It is concluded that the functional role of glutamine is complex and inadequately understood. Although it may serve as a means of storing amino nitrogen or of detoxifying ammonium ion, it would appear that it has a more vital function in intermediary metabolism as is perhaps involved in the transfer of amino groups.

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PART II. AMMONIA TOXICITY AND DETOXICATION

INTRODUCTION

The problem of ammonia toxicity is one of considerable current interest growing out of the wide-spread use of urea and various ammoniated products as non-protein sources of nitrogen in rations of ruminant species. Under certain conditions, animals receiving high levels of these non-protein nitrogenous supplements develop nervous abnormalities and may die. It is believed that, in the case of urea, the enzymatically catalyzed hydrolysis of this compound to yield ammonia and carbon dioxide proceeds at a rate in excess of the ability of the bacterial flora of the rumen to utilize it in the synthesis of amino acids and other metabolites. As a result the excess ammonia is absorbed by the animal tissues in sufficient quantities to produce toxic symptoms. Other ammoniated products, being potential sources of ammonia, as a result of chemical and or bacterial action, could conceivably result in a similar situation.

The possible relationship of these studies to glutamine metabolism will be recognized, as it has been suggested by numerous workers in this field that glutamine is involved in the translocation and storage of ammonia. The presence of glutaminase enzymes, capable of hydrolyzing glutamine into glutamic acid and ammonia, has been reported in several tissues, i.e., kidney, liver, brain, and spleen, of various species. It has also been demonstrated that under favorable circumstances the enzymatic synthesis of glutamine from glutamic acid and ammonia can be accomplished in vitro.

Of considerable significance are the findings of Sapirstein (1), who demonstrated the effectiveness of glutamic acid in lessening the toxicity of ammonia as NH_4Cl injected intravenously in rabbits.

Other indications of the importance of ammonia toxicity are apparent as it has been repeatedly affirmed in the literature that there is an increase in the concentration of ammonium ion in the brain and cerebrospinal-fluid of humans and animals in states of increased excitability. Among the symptoms associated with ammonia toxicity are hyperirritability, convulsive seizures, and muscular tetany. These symptoms, being very similar to those associated with epilepsy, and other spontaneous seizures, suggest a possible relationship. At present there is no suitable antidote known for ammonia toxicity. Any information relative to the cause of the toxic effects of ammonium ion and means of ameliorating its effects would be of immediate value to the livestock industry and might eventually have applications in human medicine.

The purposes of these investigations were two-fold: (1) to further elucidate the cause of ammonia toxicity, and (2) to find suitable antidotes for the relief of the toxic symptoms.

REVIEW OF THE LITERATURE

A review of the literature pertaining to these investigations will for the sake of simplicity be divided into three major divisions: (1) the physiological response to ammonium ion, (2) cell membrane permeability, ion antagonisms and ion imbalances involving NH_4^+, and (3) the relation of ammonia to metabolic processes in the animal organism. A search of the literature has disclosed an extremely large number of publications related to each of these subjects. Because of the large number of papers, this review will of necessity be limited to those papers which have a direct bearing on the investigations, and in certain instances only a few of the papers dealing with particular topics will be cited.

I. Physiological response to ammonium ion

A. Effect of ammonia on acid-base equilibrium.

Venulet and coworkers (2) demonstrated that 1 cc. of 10% NH_4OH given orally to a dog in milk or water, or 0.5 cc. diluted and injected intravenously, caused a decrease in the alkali reserve. One cc. inhibited the increase in alkali reserve produced by 2 gm. $NaHCO_3$. In another paper the same authors (3) demonstrated that oral administration of NH_3 leads to acidosis and counteracts the alkaline tide normally observed after a meal. It will likewise offset the increase in alkaline reserve caused by the administration of $NaHCO_3$. They claim that repeated injections of NH_3 even caused a fall in blood pH which persisted for about a week. Venulet (4) claimed that prolonged peroral or intravenous administration of ammonia resulted in a decrease in blood base which persists long after the ammonia is withdrawn. In human subjects in addition to this effect, the alveolar tension of CO_2 is decreased. They ruled out

any ventilation alkalosis by conducting spirometric tests. They conclude that although ammonia is a base, it has an acid effect on the organism and hypothesize that it may be useful in therapeutics. Alwall and Geiger (5) likewise, state that NH_4OH, when introduced into the stomach, causes a marked decline in the alkali reserve. They claim this acidosis is absent after infusion of NH_4OH into the intestine. Hazard and Vaille (6) showed that the intravenous injection of about 0.1 gm./kg. of NH_4Cl, $(NH_4)_2CO_3$ or NH_4OH caused a small decrease in the alkali reserve in rabbits, and in 13-100% increase in glucemia. Bossani and Ferrante (12) state that dogs can tolerate 4 times the m.l.d. of ammonia if the alkali reserve is maintained. They claim that oxidizable anions favor the conversion of NH_3 to urea insofar as they permit the readjustment of this alkali reserve. This depends upon the rapidity of oxidation of the anions.

B. Normal and toxic levels of ammonia in various tissues.

In experiments with sheep, Dinning and coworkers (7) demonstrated a rise in urea and NH_3 in portal blood when 40 gm. of urea in water solution was administered orally. Portal blood ammonia values continued to increase during a 2-hr. observation and reached 8.4 mg./100 ml of blood. The sheep was under light anesthesia. This indicated hydrolysis of the urea in the rumen and the absorption of large quantities of NH_4^+. When urea was administered to steers as a drench amounts in excess of 100 gm. produced a rapid rise in both urea and NH_3 in systemic blood. Ataxia appeared when NH_3-N of systemic blood reached 2.5 mg.% and symptoms of alkalosis, followed by death, occurred at about 4 mg.%. McDonald (10) claims that in the systemic blood of the sheep ammonia occurs only in a trace if at all whereas venous blood draining the rumen normally contains about 1.5 mg.% of NH_3. It was demonstrated by Mirer and Rubel (8) that arterial blood contains practically no NH_3 whereas venous blood and

blood leaving the brain contain 0.04-0.27 and 0.1-0.32 mg.% respectively. Winkelnkemper (9) administered urea, ammonium salts and acid salts to dogs via stomach tube and measured blood levels of ammonia. He reported that urea did not increase blood NH_3 and produced no effect on the central nervous system. Some acid salts as KH_2PO_4 and NaH_2PO_4 caused a transient rise in blood NH_3. Salts of NH_4^+ caused an increase, NH_4Cl causing two peaks, in blood ammonia about 40-50 minutes apart. The latter peak was thought to be due to the effect of the Cl^-. Richter and Dawson (11) state that the normal level of ammonia in rat brain is 0.28 mg.%. This level was decreased by prolonged nembutal narcosis, increased by conditions which enhance cerebral irritability, and was unaffected by emotional excitement. They further stated that when brain NH_3 level reached 9 mg./ as a result of NH_4Cl injection, convulsions ensued. Bassani and Ferranti (12) demonstrated that the m.l.d. of NH_4Cl in the dog is 150 mg./kg. body weight. They state that death is caused by exhaustion of the regulatory mechanisms of the blood reaction. Accumulation of NH_4^+ in the blood is a sign of failure to transform it into urea. Koprowski and Uninski (13) observed that the normal blood NH_3 level for resting dogs is either zero or below the analytical level. Oral administration of NH_4Cl (0.5 gm./kg.) caused a marked increase in blood NH_3. Highest mean value of 1 mg.% was observed 15 minutes after administration returning to normal in 90 minutes. A lag in urea formation of 60-90 minutes was noted, the peak in urea being noted after the blood NH_3 had returned to normal. Karr and Hendrics (14) state that toxicity of ammonium salts in dogs depend upon the rate of injection rather than the total amount injected when these salts are injected into the femoral vein. The salts used were the chloride, carbonate, acetate, and bicarbonate, in concentrations of 2, 2-2.5, 2.96, and 2.88% respectively. They reported toxic symptoms with rates as low

as 2.3 times the usual clinical rate of 0.045 milliequivalents of NH_4^+/kg./min. Schmidt and Vallencourt (15) report the results of inhaling NH_3 fumes for 4 hours at a concentration of 530-560 p.p.m. They report an increase in blood ammonia (0.0 to 36.4 mg.%) and an increase in non-protein nitrogen (27.0 to 57.0 mg.%). They were unable to detect a change in urea or creatinine, or in the CO_2 combining power of the plasma. They reported a drop in blood pressure. Ting (16) commented on the paper by Schmidt and Vallencourt. He called attention to the paper of Tauber and Kleiner (17) who reported deaths in rabbits from ammonia toxicity at NH_3 levels of 2-3.5 mg.% in the blood, and to the work of Kirk and Summer (18) who caused deaths in rabbits by intravenous injections of urease enzyme. They reported severe convulsions at blood ammonia levels of 4 and 5 mg.%. Ting (16) stated that he has conducted numerous experiments with white rats and found that when ammonium citrate is injected intraperitoneally the animals die when the blood ammonia nitrogen level of 8-11 mg.% is reached. When crystalline urease is injected the animals die at the same ammonia level. Ting is convinced that an ammonia level of 36.4 mg.% is unattainable without death ensuing.

Bruhl (19) found that normally ammonia is not detectable in cerebro-spinal fluid. Under conditions of hyperirritability it may reach 90 ± 10γ%. In convulsive states it may be as much as 450γ%. Harris, (20) reported that no ammonia was found in the cerebro-spinal fluid of 13 patients with nervous and mental diseases, but in 21 others the ammonia ranged from 0.01 to 0.02 mg./ 100 ml.

C. Miscellaneous physiological effects.

Ajmone-Marsan et al. (21) demonstrated that injections of suitable doses of NH_4Cl in lightly anesthetized dogs were either only slightly effective or sufficient to cause cardiac arrest. Sub-lethal doses were

accompanied by a conspicuous drop in blood pressure. They also detected modifications of the spinal cord activity which were independent of circulatory changes, and which consisted of a simple activation or an activation accompanied by synchronization of the spinal rhythms. They thought this was possibly due to an increase in the number of active neurons and in their degree of synchronization. They concluded that NH_4Cl "fits" were not of the epileptic type.

Balbo (22) reported a decrease in the number of erythrocytes and in hemoglobin content which appeared to be proportional to the degree of ammonia poisoning in rabbits which were given 50-80 cc. of 0.5% NH_4OH by stomach tube. Cellular elements of the red marrow were destroyed. As a later effect, myeloid elements disappeared and the number of lymphocytes increased.

MacKay et al. (23) reported an acute pulmonary edema following administration of NH_4 salts to guinea pigs which could be prevented by adrenergic blocking agents. They observed a less marked lung edema in rabbits. A central nervous system depressant (continuous ether anesthesia) also prevented the edema. This suggests that adrenergic stimuli of reflex or direct central stimulation plays a part in its production. They observed that some strains of rats and mice are resistant to production of pulmonary edema by NH_4^+.

II. Physiological Role of Magnesium Ion and Ion Antagonism.

In our search for ammonia detoxicants it was discovered that magnesium salts had a beneficial effect. The mortality rate was somewhat reduced when magnesium salts were administered simultaneously with the ammonium salts. It was postulated that magnesium ion might have an antagonistic effect on ammonium ion or that the toxic effects of ammonia might be, in

part, a result of an ion imbalance which was remedied by Mg^{++} administration. A search of the literature on the physiological effects of magnesium revealed a plethora of publications which were possibly related, one way or another to the problem. Only a small representative sampling of these publications can be cited here.

Several references were found which indicated an antoganism exists between NH_4^+ and Mg^{++} under certain physiological conditions. Prawdiez-Neminski (24) showed that NH_4OH and NH_4Cl increased the periodic movement of the empty gastro-intestinal tract and that Mg behaved antagonistically to them. Tziganov (25) stated that in limited doses Mg^{++} is antagonistic to the stimulating effect of NH_4^+. In later studies Pravdich-Neminskii (26) reported that the toxic dose of NH_4OH for Rana temporaria (frog) is 320-40 mg./kg. (as NH_3). He claimed that the administration of NH_4^+ resulted in $MgNH_4PO_4$ crystallization within the tissues. He administered $MgCl_2$ solution almost simultaneously with NH_4OH and obtained as high as 80% survival even after a definitely lethal dose of NH_4OH.

Steel (27, 28) showed that the parenteral administration of $MgSO_4$ in dogs resulted in marked increase in urinary elimination of nitrogen as NH_3, urea, creatinine and allantoin. He also observed an increased elimination of $MgNH_4PO_4$.

In several physiological phenomena there appears to be an antagonism between magnesium and calcium ions. This particular property of magnesium ion has no fundamental bearing on the effect on ammonium ion, however, the observation of these effects further emphasizes the complexity of the intricate ion balances established in various tissues, and the importance of these in physiological processes. Among the more important of the observed Mg-Ca antagonisms is that associated with the transmission of nervous impulses. Bryant et al. (29) stated that it is the Mg/Ca ratio

which is important for the functioning of the central and peripheral nervous mechanisms. In the paralyzing effect of hypermagnesia, Baumecker (30) observed that this magnesium effect is not due to the deionization of calcium but is explained by the replacement of calcium by magnesium at the motor end-plate. The antagonism by calcium, of the magnesium effect, depends on the replacement of calcium, necessary for the function of the end-plate, which has been substituted by magnesium. In connection with these observations, it is interesting to note that other anions which precipitate calcium, such as oxalate, will augment the effect of magnesium. Hoff et al. (31) studied the effects of various concentrations of magnesium on neuromuscular transmission and various reflexes in the cat. They concluded that the greater the frequency of stimulation, the higher is the concentration of magnesium necessary to block transmission. They stated that it is probable that magnesium effects muscular activity by blocking neuromuscular transmission rather than by means of its depressent action on the central nervous system. Because of this characteristic of magnesium there have been wide-spread investigations into the possibilities of using magnesium salts as general and local anesthetics, and considerable use has been made of magnesium anesthesia in both veterinary and medical practice.

Related to the foregoing property of magnesium is its use in the treatment of various forms of tetany and convulsions. Meltzer and Auer (32) claimed that intraspinal injections of $MgSO_4$ were capable of abolishing for 24 hours or more all clonic contractions in human tetanus and experimental tetanus in monkeys. However, Dixon and Ranson (33) found that $MgSO_4$ had no effect in relieving the tetany in rats injected with tetanus toxin. NH_4Cl proved to be of some benefit. Greenberg and Aird (34) report normal Mg^{++} and Ca^{++} levels in serum and spinal fluid of 28

patients with epilepsy. On the other hand, Hirschfelder and Haury (35) reported that during epileptic convulsions, plasma Mg^{++} was low and potassium content increased. They found continuous oral administration of magnesium to be of no benefit, nor did potassium chloride aggravate the siezures. Alton and Lincoln (36) recommend intraspinal injections of $MgSO_4$, and Lazzard (37) intravenous injections of $MgSO_4$ to abolish convulsions associated with puerperal eclampsia. Miller (38) observed tetany in a 6 year old child associated with osteochondrosis of the capital epiphysis of the femur. Tetany developed when plasma magnesium was 0.6 and 1.7 mg.%. Oral administration of $MgSO_4$ increased plasma magnesium to 2.6 mg.% with a concomitant improvement in nervous hyperirritability. McGhee (39) observed that canine-hysteria was due to deficiency of magnesium in the blood. Spak (40) treated dementia praecox by injections of hypertonic $MgSO_4$ solutions (5 gm. of 20% solution) in combination with scopolamine. Magnesium deficiency has also been associated with grass staggers and lactation tetany in cows. Hapkirk (41) claimed that grass staggers in dairy cows could be overcome by increasing the blood magnesium through the addition of dolomite to the feed. Cunningham (42) stated that animals effected by grass staggers have low magnesium content in their blood and respond to treatment with magnesium. Aston (43) found the blood of cows suffering from grass staggers to be markedly deficient in magnesium and showed that the magnesium content of the diet was reflected in the blood-magnesium level. Blood magnesium was raised to above normal by feeding extra magnesium as sulfate, carbonate, chloride or phosphate. Duncan, Huffman and Robinson (44) report that calves cannot be raised to maturity on whole milk rations because they develop tetany associated with low blood magnesium. This tetany resembles low calcium tetany and can only be distinguished from it by blood analysis. The

blood Ca to Mg ratio was 8:1 to 10:1 in calves showing tetany. Blood calcium was normal but blood magnesium was low. Lactation tetany in cows has been attributed to low blood magnesium by Blackmore and Stewart (45) and to low calcium and magnesium by Nicholson and Shearer (46). Green et al. (47) state that hypomagnesia and hypocalcia are characteristic of equine transit tetany.

Greenberg and Tufts (48) describe the nature of magnesium tetany. They conclude that the lesion associated with hyperirritability is localized in the midbrain or pons, as they noted an increased sensitivity to the convulsant action picrotoxin and the protective action of subanesthetic doses of sodium amytal against convulsions. They observed a difference in calcium tetany and magnesium tetany. Curare does not prevent the onset of convulsive siezures in the latter type. Of particular interest was a paper by Caldwell and Hughes (49) who supported the hypothesis that nerve tissue is an emulsion-like organization of lipin and non-lipin components which respond to the relative concentrations of mono- and divalent ions in a manner similar to the response of water and oil emulsions in vitro. They think sodium, potassium, calcium and magnesium are most likely to be involved. They call attention to the fact that potassium and magnesium are present in relatively large amounts in the tissues whereas sodium and calcium are more abundant in extracellular fluids. These observations support the hypothesis of the author with regard to an interrelationship of NH_4^+ and Mg^{++}, as it is well known that NH_4^+ is very similar in many properties to K^+, e.g., they form isomorphous crystals, the ions both have a single positive charge, they are usually solvated to about the same degree, their compounds have similar solubilities, and the ions have almost identical physical dimensions. It is reasonable to assume some similarity in physiological effect to these similar ions. Kirschfelder (50) held

similar views. He noted that the anesthesia produced by injections of magnesium salts into rabbits can be instantly relieved by injections of univalent ions. He gives the order of effectiveness, however, as K, Rb, Cs, Na, Li, NH_4. He suggests the theory of a balance of emulsions in the surface layer of the cells. Smith (51) noted a similarity in paralytic symptoms induced by high magnesium and low potassium blood levels in dogs and investigated the possibility of a relationship. She found that the increase in magnesium level of serum following parenteral injections of $MgSO_4$ was accompanied by a fall in serum potassium.

III. The Interaction in vivo of α-keto Acids with Ammonium Ion.

In our studies of detoxicants for ammonia, experiments were performed using citrate and acetate salts. It was noted that there appeared to be a slight difference in the toxicity of ammonia when administered in these two forms. It was decided to investigate various anions, particularly those known to be involved in ammonia metabolism, i.e. several α-keto acids anions, as possible antidotes for ammonia poisoning. A review of the literature uncovered many publications related to the problem. A few will be cited to establish a theoretical background for the results of the investigations herein described.

Rose and coworkers (52) demonstrated that rats which were supplied with the 10 essential amino acids in their diet showed accelerated growth when ammonium salts, L-glutamic acid, glycine, or urea was added to the diet. Both growth tests and nitrogen balance studies on these animals demonstrated that ammonium salts can be utilized by the rat under the conditions used. Lardy and Feldott (53) also demonstrated that young growing rats would continue to grow when fed the essential amino acids if ammonium salts were fed. Ammonium citrate appeared to effect better

growth than a supplement of nonessential amino acids on an arginine-free diet, and was equally well utilized by rats receiving all the essential amino acids. The classical experiments of Schoenheimer's group (54) could be cited, also, as direct evidence that ammonia is utilized by monogastric species. They administered $N^{15}H_3$ as ammonium citrate and demonstrated the isotope in numerous amino acids, creatine, and other compounds. Krebs, Eggleston and Hems (61) demonstrated the synthesis of glutamic acid by liver homogenates from NH_3 and pyruvate, oxalacetate, α-keto-glutarate, or citrate. This synthesis proceeded aerobically but more rapidly under anaerobic conditions. Sprinson and Rittenberg (55, 56) have extended similar findings to humans and by use of labeled ammonium citrate and glycine have been able to measure the actual rate of protein synthesis. Wiss (57) likewise observed the synthesis of alanine from pyruvic acid and NH_3 by liver slices. Kritzman (58) stated that the synthesis of amino acids in surviving liver slices probably involves the following steps: (1) formation of oxalacetate from CO_2 and pyruvate, (2) formation of aspartic acid (or glutamic acid) from oxalacetate (or α-ketoglutaric) acid, ammonia, and an unidentified donor, and (3) formation of alanine by transamination between pyruvate and aspartic (or glutamic) acid. Kritzman and Melik-Sarkisyan (59) give additional evidence for the indirect synthesis of alanine (involving a dicarboxylic acid) through the study of amino acid formation by liver and kidney slices in the presence of pyruvate, oxalacetate, and α-ketoglutarate. The individual amino acids formed were determined. A short period of incubation of liver slices with pyruvate, NH_4Cl, buffered with bicarbonate at pH 7.4, yields largely aspartic acid with a small amount of alanine. The same products were formed from pyruvic acid, NH_3, and CO_2, with the same speed as from oxalacetic acid. The formation of amino compounds from

α-ketoglutarate and NH_3 proceeded more slowly. Short periods of incubation of α-ketoglutarate resulted in the accumulation of aspartic acid and not glutamic acid.

These few references, though by no means complete, will serve to justify our findings that certain anions have a beneficial effect in lessening the toxicity of ammonia. There is ample proof that ammonia is used by animals in the synthesis of amino acids and other compounds. It is not unreasonable to assume that supplying the precursors for these syntheses in the form of α-keto anions could result in an increase in the rate of these reactions, the net result being to reduce the concentration of ammonia in the blood.

EXPERIMENTAL

Preparation of Solutions of Ammonium Salts

In all cases except ammonium acetate, the solutions were made by adding the purified acid to a NH_4OH solution, which was slightly more concentrated in NH_4^+ than required, until a pH of 7.4 was attained. The NH_4^+ concentration was then measured either by nesslerization or by a semi-micro Kjeldajl procedure (without preliminary digestion). (The former method was found to be less accurate as several dilutions were required and there was some evidence of anion interference in certain cases.) The solution was then diluted to the desired concentration and the NH_4^+ content re-checked. In the case of the ammonium acetate the crystalline salt was weighed out directly, diluted to volume and the pH adjusted to 7.4 with acetic acid. The concentration was checked by analysis for NH_4^+.

Animals

The animals used were rats of both sexes of the Sprague-Dawley strain from the local colony. They were all fed the stock ration used for the colony *ad libitum*. Extremes in size and age were avoided, the average weight of the animals used being about 200 gm. Only animals in sound health and a good state of nutrition were used. They were assigned to treatment at random. They were weighed to the nearest 10 grams and the ammonium salts were administered intraperitoneally on a weight basis.

Glutamate as a detoxicant for ammonia

Sapirstein (1) reported the effectiveness of glutamic acid in lessening the toxicity of ammonia injected intravenously in rabbits. He

suggested the interaction of ammonia with glutamate to form glutamine as the probable reason for the effect. Tigerman and MacVicar (60) found that the simultaneous administration of glutamic acid and ammonium ion resulted in an increase in glutamine content of the tissues in rats. It was deemed of interest, therefore, to determine the effectiveness of glutamic acid as a detoxicant for ammonia in the rat. Ammonium citrate was chosen as the form in which the ammonium ion for the control group would be administered. Solutions of ammonium citrate and ammonium glutamate were prepared and administered to rats by intraperitoneal injection. The toxic level of the ammonium ion administered as the citrate was established and then this was compared to ammonium ion administered as glutamate. Ammonium citrate and glutamate were injected at three levels, 100, 150 and 200 mg./kilo. The results are shown in Table 1.

Table 1

Results of Intraperitoneal Injections of Ammonium Citrate and Ammonium Glutamate in Rats

Salt Administered	mg. NH_3/kilo	No. of Animals	No. Survived	Percent Mortality
NH_4 citrate	100	3	3	0
NH_4 glutamate	100	3	3	0
NH_4 citrate	150	25	21	16
NH_4 glutamate	150	26	11	58
NH_4 citrate	200	10	2	80
NH_4 glutamate	200	10	0	100

The symptoms of ammonium ion toxicity were observed to be as described in the literature. The first effect noted was an apparent drowsiness which took effect almost immediately, followed by convulsions, coma and death. There was evidence of respiratory involvement as, in severe cases,

the breath came in short gasps becoming less frequent as the symptoms progressed. The animals showed a marked hyperirritability, any sharp sound or external stimulus provoking convulsive muscular response. Frequently the animals expired in a state of violent tetany which lasted for several seconds, death usually occurring within 45 minutes after administration of the salt, and frequently as soon as 10 minutes at the higher levels. Animals surviving for an hour, almost without exception, recovered. Recovery seemed to be complete judging from appearance of the animal, however, animals almost always succumbed to a second injection even after an interval of a week or more, suggesting tissue damages. The convulsive and tetanic symptoms appeared to be less severe in those animals receiving ammonium glutamate.

It will be observed from Table I that the mortality rate was considerably higher among those animals receiving the glutamate salt than the citrate. The values given for the 150 mg./kilo level are a composite of three trials. In each case the mortality was higher in the group receiving glutamate.

Some of the tissues were removed from the animals used in the third trial at the 150 mg./kilo level. These were assayed for glutamine in the manner described in Part I. The results of this analysis are shown in Table II.

It is evident that there is no increase in the glutamine level of liver and kidney tissues resulting from the administration of ammonium glutamate as compared to those receiving ammonium citrate. These values are in the range of normal values on the basis of previous and subsequent analyses. These findings do not agree with the data of Tigerman and MacVicar (60) although certain differences in techniques and in amount of NH_4^+ administered existed between the two experiments.

Table II

Tissue Glutamine Levels in Rats Following Intraperitoneal Injections of Ammonium Citrate and Ammonium Glutamate

	Citrate	Glutamate
Tissues	Glutamine (mg.%)*	
Liver	67	65
Kidney	76	57
Number of animals	7	9

*Results obtained by distillation and nesslerization

In view of these results it was thought that possibly giving glutamate in advance to the administration of ammonium citrate might give different results, as the glutamate would already be translocated to the site of activity and possibly the mode of combination of glutamate with ammonia to form glutamine would already be in operation. A solution of sodium glutamate was prepared by neutralizing L-glutamic acid with NaOH to a pH of 7.4, and contained 17.6 milli-equivalents of glutamate/kilo body weight. This is 1 1/2 times the stoichiometric level of NH_3 at the 200 mg./kilo level. This injection to 16 animals was followed in 30 minutes by an injection of ammonium citrate at the 200 mg./kilo level. An additional group of 12 animals received the citrate injections to serve as a control group. The results of this trial are shown in Table III.

It will be noted that the mortality rate for the controls was 67% while the preinjected group all died. The average time of survival was notably less in the preinjected group.

On the basis of these experiments it must be concluded that glutamate, when injected simultaneously or immediately preceeding the injection of ammonium ion does not lessen the toxicity of this substance to the rat.

Table III

Results of Preinjection of Glutamate Solution Upon the Toxicity of Ammonium Ion Administered as Ammonium Citrate by Intraperitoneal Injection

	Group I, Preinjected with Na Glutamate	Group II Control
Number of rats	16	12
Number of survivors	0	4
Percent mortality	100	67
Avg. time of survival, (min.)	23.3	31.3

On the contrary, the evidence strongly suggests that the toxicity is actually enhanced by the addition of glutamate. It may be that glutamate is metabolized with release of amino groups which add to the NH_4^+ administered parenterally, to a greater extent than it is combined with ammonia to form glutamine. In any event, the evidence at hand does not support the use of glutamate as an antidote for ammonia poisoning.

The Effect of Sodium on Ammonium Toxicity

It came to our attention, about this time, that a deficiency of plasma calcium could cause tetanic conditions similar to those observed resulting from administration of ammonium ion. As citrate was being used as the anion in our control groups, and as citrate ion is known to form a complex with calcium ion in the blood rendering is ineffective in its physiological roles, it seemed advisable to change to another anion to avoid the possible implication of the citrate in these studies. Acetate was chosen as an anion because it is easily metabolized and a search of the literature revealed no known implication with any of the cations that would interfere with our results. It thus became necessary to

establish the toxic level of ammonium ion when administered as acetate. Table IV shows composite results of a number of trials at different levels of concentration. From it a fair picture of the toxicity of ammonium acetate is obtained.

Table IV

Toxicity Level of Ammonium Acetate in the Rat

NH_4^+ level mg./kilo	No. of Animals	Mortality	Percent Mortality	No. of Trials Included
100	2	0	0	1
132	10	5	50	1
150	131	90	69	11
200	5	5	100	1

By comparison with Table I it will be noted that ammonium acetate was more toxic than ammonium citrate. It seemed to have about the same level of toxicity as ammonium glutamate.

Ammonium ion is known to have a powerful hemolytic action. It is supposed that it enters the erythrocyte producing a marked hypertension which results in rupture of the stroma with liberation of the hemoglobin. One would expect this because of the close similarity of ammonium ion to potassium ion in physical properties, and potassium ion is considerably more concentrated in the cells than in the plasma. This suggested that part of the toxic effects of ammonium ion might be engendered by its hemolysin effect. It was postulated that an ionic balance could be reestablished by injecting sodium ion, which would largely remain in the plasma, producing a tension which would offset that created within the cell by the enterance of ammonium ion. An experiment was conducted, accordingly, in which ammonium ion was administered at the level of 150 mg./kilo and sodium at the level of 200 mg./kilo, both as acetate salts. This is roughly

the relative ionic strength of potassium to sodium in the plasma. The results of this experiment are shown in Table V. The composite value previously listed in Table IV, for the mortality of ammonium acetate injected animals is used as a comparison, though a control group consisting of 10 animals injected at the same time had a 50% survival.

Table V

Effect of Sodium on Ammonia Toxicity

NH_4^+ Level mg./kilo	Na^+ Level mg./kilo	No. of Animals	Mortality	Percent Mortality	No. of Trials Included
150	----	131	90	69	11
150	200	15	12	80	1

It is readily seen from these data that sodium ion, under the conditions administered, offered no protection against the toxicity of ammonium ion. The data strongly suggest that mortality was increased somewhat by administration of Na^+.

Effect of Divalent Ions on Ammonia Toxicity

As sodium ion appeared to have a detrimental effect upon the toxic action of ammonium ion it was decided that this action might be because both are univalent ions. It is well known that many divalent ions will reverse the phase on an oil-in water emulsion. Cytologists have postulated the possibility of lipin and non-lipin phases play an important role in the physiological processes of cells. It is supposed that the balance between mono- and di-valent ions is important in maintaining desirous cell permeabilities and numerous pathological conditions have been ascribed to improper balance of these ions (49, 50, 51). Because of the similarity in symptoms of ammonia toxicity and "grass staggers", and as

it has been reported that this malady is remedied by administration of Ca^{++} or Mg^{++}, (42, 43), it was thought that the toxic effects of ammonium ion might in part be due to an ion imbalance of the mono- and di-valent type. Consequently it was considered expedient to determine the effect Ca^{++} and Mg^{++} on the toxicity of NH_4^+.

Solutions were made containing ammonium ion at the level of 150 mg./kilo and calcium and magnesium ions at varying levels, all as acetate. Table VI summarizes the results of these experiments.

Table VI

Effect of Divalent Ions on Ammonium Toxicity

NH_4^+ Level mg./kilo	Additional Ions, mg./kilo	No. of Animals	Mortality	Percent Mortality	No. of Trials Included
150	none	131	90	69	11
150	Ca^{++}, 6	19	11	58	2
150	" , 48	9	5	56	1
150	Mg^{++}, 4.8	15	8	57	1
150	" , 9.6	15	6	40	1
150	" , 19.2	81	30	37	6
150	" , 38.4	21	3	14	2
150	(Ca^{++}, 48.8) (Mg^{++}, 19.2)	13	6	46	1

It will be noted that there was slight improvement when calcium ion was administered simultaneously with ammonium ion. The administration of magnesium ion showed a larger effect. This effect is more pronounced as the concentration of the magnesium ion is increased. The combination of calcium and magnesium gave no advantages over magnesium alone.

Effect of anion on ammonia toxicity

The experiments conducted with citrate, glutamate and acetate indicated that there is an anion effect (compare Tables I and IV). Glutamate and

acetate appeared to act about the same whereas citrate appeared somewhat less toxic. As ammonia is known to be utilized by the rat in the synthesis of amino acids (52 through 59) it seemed logical to assume that the precursors of amino acids might have a beneficial effect upon an animal suffering from ammonia toxicosis. Three α-keto acids known to be readily converted to amino acids by reductive amination, viz., pyruvic, oxalacetic and α-keto glutaric acids, were compared with acetic and citric at two levels. The procedure employed here was the same as described previously, i.e., the ammonium ion and the respective anions were administered simultaneously as the ammonium salt of the acid. The results of these experiments are shown in Table VII:

Table VII

Effect of Anion on Ammonium Ion Toxicity

NH_4^+ Level Mg./kilo	Anion	Additional ion, Mg./kilo	No. of Animals	Mortality	Percent Mortality	No. of Trials
132	Acetate	---	20	10	50	2
132	Pyruvate	---	20	1	5	2
132	Citrate	---	20	9	45	2
132	Acetate	Mg^{++}, 38.4 (as Mg acetate)	20	4	20	2
150	Acetate	---	131	90	69	11
150	Pyruvate	---	18	9	50	2
150	Oxalacetate	---	18	6	33	2
150	α-ketoglutarate	---	8	1	13	1
150	Acetate	Mg^{++}, 38.4 (as Mg pyruvate)	10	2	20	1

On the basis of these data it appears that the α-keto acids offer a measure of protection against ammonia poisoning. At both levels the toxicity of ammonium ion is less when administered as the salt of an α-keto acid as when given as the acetate. Here again magnesium ion, when

administered simultaneously with ammonium ion, is beneficial. Magnesium pyruvate appeared to be more beneficial than magnesium acetate on the basis of one trial. The percent mortality was the same with the level of ammonium ion being higher when the magnesium pyruvate was administered. These results should not be considered as conclusive, however, as a relatively few animals were involved and considerable difference was noted in the response of the animals.

DISCUSSION

Experiments conducted in this laboratory strongly support the hypothesis that the toxic effects of ammonium ion to animals, at least in part, lies in a disturbance of the ionic balance of the mineral constituents in various tissues. A number of observations point to this conclusion, most obvious of which are:

First, there is a striking similarity of the symptoms of ammonia toxicosis to those attributed to a deficiency of such bivalent ions as magnesium and calcium. Assuming that certain physiological processes and conditions, such as membrane permeability, conduction of nerve impulses, irritability of muscle fibers, etc., are dependent upon the maintainance of certain lipin-nonlipin phases, it is understandable how an excess of a univalent ion could disrupt these processes. It is well known that in <u>in vitro</u> systems a slight change in the ratio of univalent to bivalent ions will result in a reversal of phase in oil-in-water and water-in-oil emulsions. In these systems a deficiency of a bivalent ion causes the same phase reversal as an excess of a univalent ion.

Second, the addition of sodium ion appeared to enhance the toxic effects of ammonium ion. In light of the above, the addition of sodium ion would further shift the imbalance away from the normal.

Third, the addition of calcium ion or magnesium ion caused a definite decrease in the toxicity of ammonium ion. Magnesium ion was more effective than calcium ion. This would be expected on the assumption that ammonium ion is more like potassium ion than sodium ion in its physiological effects, as potassium ion and magnesium ion are found in the cell in relatively

greater concentrations than in the extracellular fluids. This would indicate, if ammonium ion enters the cell readily, as is certainly the case with the erythrocyte, the magnesium ion, being concentrated within the cell would be at the site of action of the ammonium ion.

It is likely that ammonium toxicity is multiple in its effect as offsetting the imbalance in ions by administration of magnesium salts resulted only in a partial remission of the symptoms.

It is well known that the body has mechanisms by which it can dispose of ammonia. Anything which can be done to speed up the rate of the mechanisms would aid materially in overcoming ammonia poisoning. Evidence is presented here to show that administration of the anions of certain α-keto acids aid in reducing the toxic effects of ammonium ion. It is supposed that an increase in the concentration of these anions materially increases the rate of utilization of the ammonium ion for the synthesis of amino acids by mechanisms already well established.

There is an indication that the simultaneous administration of magnesium ion and such α-keto acid anions as pyruvate results in an additive effect. Magnesium pyruvate would lend itself well to intravenous administration. It might prove to be an effective antidote against ammonia poisoning in large animals. Further studies along these lines seem fully justifiable.

The studies in this laboratory with rats as the experimental animal do not support the findings of Sapirstein (1) that glutamate is an effective antidote for ammonia in the rabbit. This may be due to a species difference. No obvious reason is known for the failure to observe an increase in tissue glutamine when ammonium ion and glutamate were administered simultaneously, as was shown by Tigerman and MacVicar.

SUMMARY

Glutamate ion, when administered simultaneously with or immediately preceding the injection of ammonium ion, offers no protection against the toxic effects of ammonium ion in the rat. The simultaneous intraperitoneal administration of ammonium ion and glutamate ion did not result in an increase in glutamine content of liver and kidney in the rat.

Sodium ion was ineffective in lessening the toxicity of ammonium ion, and, in fact, appeared to enhance the toxic effects. This may be due to the fact that both are monovalent ions and therefore increasing Na^+ in the body fluids aggravates an already existing imbalance between mono- and divalent ions.

Calcium and magnesium ions gave some protection against ammonium toxicity in the rat. Magnesium ion was more effective than calcium ion. This supports the theory that the toxicity of ammonium ion is at least in part due to an imbalance between mono- and divalent ions.

Certain anions, particularly those which are precursors of amino acids through amination, viz., pyruvate, oxalacetate and α-keto glutarate, likewise offered some protection.

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