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To the Graduate Council:

I am submitting herewith a dissertation written by Grace Kathleen Newell entitled "The Effects of Age and Acid Stress on Bone and Renal Responses of Rats." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

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THE EFFECTS OF AGE AND ACID STRESS ON BONE AND RENAL RESPONSES OF RATS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Grace Kathleen Newell

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ABSTRACT

The effects of age and acid stress on bone and renal responses in rats were investigated. Albino, male weanling rats and male 10-month old retired breeder rats were maintained on a commercial laboratory chow until they were 4 months and 16 months of age, respectively, and then transferred to semi-synthetic experimental diets, containing either 500 mg or 100 mg calcium per 100 g diet, with or without 2% ammonium chloride as an acid stress. The rats remained on the experimental diets 9 months, that is, until the "young" animals were 13 months and the "old" ones were 25 months of age. Prior to sacrifice, 72-hour urine collections were made on all rats for the determinations of urine volume, pH, titratable acid, ammonium, calcium and phosphorus excretions. At the time of sacrifice, blood samples were taken for determination of serum calcium and phosphorus concentrations, kidneys were weighed and their phosphate-dependent glutaminase activities and concentrations of proteins were measured. The right tibias were removed for determination of their dry and fat-free weights, and they were also analyzed for their percent ash, calcium and phosphorus.

As measured in this study, neither organic nor inorganic phases of tibias were affected by dietary treatments in either age group of rats, but tibias of old animals weighed significantly less and their tibia fat content was significantly higher than that of younger animals. Serum phosphorus levels were significantly higher in young rats.

Acid-stressed animals showed significant decreases in urinary pH and significant increases in urinary titratable acid, ammonium, calcium and phosphorus excretions, with age-associated decrements in those urinary responses in old rats. The urinary responses were affected by the level of calcium in the diet in both age groups. Renal hypertrophy was observed in acid-stressed animals, as well as significantly increased phosphate-dependent glutaminase activities; however those changes were not age-associated. Concentrations of kidney protein were significantly lower in old rats than in younger animals, regardless of dietary treatments. The effects of incorporating sodium and potassium bicarbonate into the diets containing ammonium chloride were most pronounced in old animals fed the low calcium diet as evidenced by significant decrements in the urinary responses. The data indicated that animals in both age groups adapted to the acid stress and decreased level of calcium in the diet.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	3
	Effects of Dietary Calcium Level, Calcium to Phosphorus Ratio and Age on Bone	3
	Renal Response to Metabolic Acidosis	9
	Bone Response to Metabolic Acidosis	13
	Age-Related Changes in Renal Function	19
III.	EXPERIMENTAL PROCEDURE	28
	General Plan	28
	Diets	29
	Methods	35
	Urine collection	35
	Determination of urinary pH and titratable acid	36
	Determination of urinary ammonia nitrogen	36
	Ashing of urine samples and preparation of ash solutions	38
	Determination of urinary calcium	39
	Determination of urinary phosphorus	41
	Sacrifice of animals	42
	Determination of phosphate-dependent glutaminase	43
	Determination of kidney protein	46

CHAPTER PAGE

Serum calcium determination 48
Serum phosphorus determination 50
Removal and fat extraction of tibias 52
Determination of tibia ash and preparation of ash solutions 53
Determination of tibia ash calcium 54
Determination of tibia ash phosphorus 55
Statistical methods 57
IV. RESULTS 58
Feed Intakes, Body Weights and Weight Gains 58
Renal Responses 66
Serum Responses 81
Bone Responses 84
V. DISCUSSION 90
Renal Adaptation 90
Diet Differences 94
VI. SUMMARY 101
REFERENCES 103
VITA 113

LIST OF TABLES

TABLE PAGE

1. Animal Groups and Overall Dietary Treatment 30
2. Composition of Experimental Diets 31
3. Composition of Salt Mixtures 32
4. Levels of Minerals in the Diet Compared with Levels Required for Growth of Rats 33
5. Variable Composition of the Experimental Diets as Provided by the Acid-Base Mixture 34
6. Analysis of Variance of Mean Daily Feed Intakes 59
7. Effects of Dietary Treatments on Mean Daily Feed Intake at Selected Weeks on Experiment 60
8. Effect of Age on Mean Daily Feed Intake at Selected Weeks on Experiment 61
9. Analysis of Variance of Mean Body Weights and Mean Weight Gains 62
10. Effects of Dietary Treatments on Mean Body Weights and Mean Weight Gains 63
11. Effect of Age on Mean Body Weights at Selected Weeks on Experiment and Weight Gain 65
12. Analysis of Variance of Urinary Volume and Five Urinary Parameters 67
13. Effects of Diet Within Age Groups on Five Urinary Parameters . 68

TABLE PAGE

14. Effect of Age Within Diet Groups on Five Urinary Parameters 70

15. Effects of Dietary Treatments on Urine Volume 75

16. Analysis of Variance of Three Kidney Parameters 76

17. Effects of Dietary Treatments on Kidney Parameters 77

18. Effect of Age on Kidney Parameters 79

19. Analysis of Variance of Serum Calcium and Phosphorus 82

20. Effects of Dietary Treatments on Serum Calcium and Phosphorus 83

21. Effect of Age on Serum Calcium and Phosphorus 85

22. Analysis of Variance of Tibia Parameters 86

23. Effects of Dietary Treatments on Tibia Parameters 87

24. Effect of Age on Tibia Parameters 89

CHAPTER I

INTRODUCTION

Diets in the Western World usually include a moderate to large amount of meat, and the urinary pHs of the inhabitants are predominantly on the acid side of neutral. It has been suggested that the increased incidence of osteoporosis with age may be due, in part at least, to life-long utilization of the buffering capacity of the basic salts of bone for the maintenance of acid-base balance. Ammonium chloride-induced acidosis in animals has been reported to result in significant loss of bone.

Studies with human beings and animals have shown that there are age-related decrements in renal function e.g. concentrating ability, inulin clearance, and para-aminohippurate transport. It has been suggested that such renal impairment may be due to loss of nephrons and/or tubular defects.

Information regarding the effects of acidosis on bone loss as related to calcium intake or to the calcium-phosphorus ratio of the diet, and to age-associated differences in the handling of an acid load is limited. It was the hypothesis of this study that acid-stressed adult rats would undergo bone losses, and that these losses would be influenced by the dietary level of calcium. Furthermore, it was hypothesized that such bone losses would be more pronounced in old animals due to the age-associated decrements in renal response to an

acid stress. Thus, the present study was undertaken to investigate the effects of dietary calcium level and ammonium chloride-induced acidosis on bone, serum and renal changes as related to the age of the animals.

Groups of young (4 months) and old (16 months) rats were fed two levels of dietary calcium, 100 mg and 500 mg/100 g of diet for 9 months with and without dietary ammonium chloride (2%) or dietary base in the form of sodium bicarbonate (0.33%) and potassium bicarbonate (0.40%). At the termination of the experimental period, urinary measurements were performed, and then the animals were sacrificed to evaluate bone, serum and kidney responses.

CHAPTER II

REVIEW OF LITERATURE

I. EFFECTS OF DIETARY CALCIUM LEVEL, CALCIUM TO PHOSPHORUS RATIO AND AGE ON BONE

The role of dietary calcium in skeletal diseases of the aged, especially senile osteoporosis, is a matter of prime interest. Nordin (1) has attributed this disease to a prolonged suboptimal intake of calcium. Smith et al. (2) reported that calcium intake of osteoporotic patients was less than that of normal controls, and Nordin (1), Lutwak and Whedon (3), and Harrison et al. (4) have demonstrated that calcium retention by senile osteoporotics can be enhanced by administering supplementary calcium. These results are in disagreement with those of Hayes et al. (5) who observed no difference in dietary habits of osteoporotics and control patients, and Bogdonoff et al. (6) who could demonstrate no increase in the calcium retention of osteoporotic patients given a calcium supplement of 1500 to 1600 mg/day.

The prolonged period of time over which osteoporosis develops in man limits experimental studies on human beings, therefore most investigators have used experimental animals. McClendon et al. (7), Menczel et al. (8), Harrison and Fraser (9) have demonstrated that a condition similar to osteoporosis can be produced in rats fed low

calcium diets from weaning and that cure can be effected by feeding normal diets.

Studies of the effect of different levels of dietary calcium on bones have generally been of relatively short duration and limited to single bones, but in an experiment lasting 320 days Menczel et al. (8) found an increase in the percentage ash content of the ends and shafts of the tibia but not of the whole femur of rats, when the level of dietary calcium was raised from 0.1 to 0.8%. Similarly, Williams, Mason and McDonald (10), using an X-ray technique, found differences in density of the ninth caudal vertebra in rats fed diets containing 0.1 to 0.5% calcium and 0.39% phosphorus from 74 days of age to death. The mean range of bone densities of rats fed 0.1% dietary calcium was 0.39 to 0.66 X-ray equivalent grams of ivory/cm^3 of bone, whereas that of rats fed 0.5% dietary calcium ad libitum was 0.73 to 0.91.

Krishnarao and Draper (11) have called attention to the marked differences among the bones of animals with respect to their susceptibility to osteoporosis. They performed physical measurements on the tibias, femurs and lumbar vertebrae of mice 6, 18, 30 and 40 months old, which had been fed a standard mouse diet containing 1.7% Ca and 0.8% P from weaning age. Their results showed that extensive thinning of the vertebrae and femoral cortex occurred by 18 months of age and by 30 months the thickness of these cortices, compared to that of the 6-month old animals, had decreased by 76% and 62%, respectively. Thinning was accompanied by an increased porosity of the cortex. The age-related changes in the tibias were considerably milder than those which occurred in the femurs and vertebrae.

Larsson (12) showed that when a low-calcium diet was fed to adult rats, which had been adequately fed during the growth period, an evident reduction of bone mass resulted after 14 to 16 weeks. The ash content of the whole tibia declined by 6.2% after 16 weeks and 13.3% after 12 months on a diet containing 0.09% calcium and 0.55% phosphorus. The calcium phosphorus ratio was 1:6. A decrease in the hydroxyproline content of the metacarpal bone and in the density of the humerus was also observed. Subnormal blood calcium values occurred at different periods during prolonged calcium restriction, which were slowly corrected by bone mineral. Tracer studies showed that in spite of adaptation mechanisms, such as increased calcium retention during the feeding of the low-calcium diet, increased bone resorption and net loss of calcium occurred.

The relationship between calcium retention and body stores has been studied extensively in the rat. In young, growing animals a period of low intake of calcium increases the retention of calcium in a subsequent period on a high calcium diet (13-16). In old rats also, the absorption of calcium has been found to be definitely increased after prolonged calcium restriction (17). Henry and Kon (16) demonstrated that aged rats fed a diet containing a liberal allowance of calcium went into negative balance when subjected to a low-calcium regimen, whereas equilibrium was attained if the animals had been previously adapted to a low calcium intake. Henry et al. (18) indicated that old rats fed a low calcium diet need a minimum of four months to adapt themselves to the low calcium intake and that this period of adaptation is longer for old than for young rats.

Benson et al. (19) studied the effect in female weanling rats of previous calcium diets on adaptation to both a low and a high calcium diet and the mechanism involved in such adaptation. Rats accustomed to low intakes of calcium, retained a larger percentage of the dietary calcium than rats accustomed to higher amounts of the element, regardless of whether the present calcium intake was low or high. Rats fed a low calcium diet (0.2%) adapted to it so that at 156 days of age their skeletons contained the same concentrations of calcium and phosphorus as rats which received a high calcium diet (0.9%). Parathyroid activity as estimated by parathyroid gland weight and urinary phosphate values was greater in rats fed a low calcium diet during two periods than in rats fed a high calcium diet followed by a low calcium diet. Their data demonstrated that the adaptation to both high and low calcium diets was affected by previous dietary calcium levels and reinforced the concept that body stores of calcium control its retention.

Kane et al. (20) observed a slow reduction of the absorption efficiency in old rats, while no change in calcium metabolism with age was indicated by the findings of Henry and Kon (15) and Nicolaysen (21). Hansard and Crowder (22) found that true digestibility of dietary calcium by rats declined from 41% at 48 to 72 weeks of age to 24% at 106 weeks, while the requirement for maintenance approximately doubled. Hironaka et al. (23) reported that aged rats (22 to 32 months) are subject to a greater loss of calcium from the body than younger rats (10 to 12 months), but that they are able to maintain calcium equilibrium by increasing the uptake of dietary calcium from the intestine.

Recent studies have focused attention on the possible influence of dietary phosphorus on the development of osteoporosis in animals. Krook and co-workers (24-26) have indicated that diets, either low or optimal in calcium but high in phosphorus, fed to young cats, horses and swine lead to increased rate of bone resorption. They have proposed that the accelerated bone resorption is due to parathyroid gland stimulation as a result of hypocalcemia following hyperphosphatemia (secondary hyperparathyroidism).

Draper and associates (27, 28, 29, 32) in a series of long-term experiments on mice and rats have reported that aging in these species is associated with osteoporotic changes in the skeleton, including cortical thinning and loss of mineral and organic matrix, which are similar to those observed in man. They have observed that these changes occur despite apparently optimal allowances of all essential nutrients including calcium, phosphorus, vitamin D and fluoride. However, the rate and degree of bone resorption are increased by high concentrations of phosphorus in the diet.

Shah et al. (27) studied adult mice fed levels of calcium from 0.1% to 2.4% at calcium to phosphorus ratios of either 1:1 or 2:1. They reported that bone loss in aged mice, as measured by cortical thickness and mineral content of the femurs, was increased either by a diet low in calcium or an adverse Ca/P ratio, such as 1:1. Their results indicated that the most favorable bone characteristics in terms of dry weight, total calcium content, and breaking load resulted with a diet containing 1.2% calcium and a Ca/P ratio of 2:1.

Anderson and Draper (28) and Draper et al. (29) have confirmed the accelerating effect of high phosphorus diets on bone resorption in aging rats by chronic labeling of the skeleton with ^{45}Ca. They demonstrated that the effect of dietary phosphorus was not due to a depression of calcium absorption. Clark (30) has reported that the absorption of calcium by adult rats fed a diet containing 0.2% of this element was unchanged over a wide range of phosphorus intakes. However, Shah and Meranger (31) found that dietary phosphorus depressed calcium absorption in weanling rats, suggesting an age effect.

Anderson and Draper (28) found bone resorption in rats caused by high intakes of phosphorus could be eliminated by parathyroidectomy. They postulated that the osteoporosis-inducing effect of dietary phosphorus is due to a mild secondary hyperparathyroidism induced by depression of serum calcium concentration occasioned by the flux of excess phosphorus through the blood.

Krishnarao and Draper (32) have investigated the reversibility of bone resorption caused by feeding a high phosphorus diet to mice from early adulthood to middle age and found that the accelerated rate of bone resorption could be arrested by lowering the phosphorus content of the diet from 1.2% to 0.6% at 18 months of age.

Anderson and Draper (28) called attention to the average Ca/P ratio in the U.S. dietary of 1:2.8 and commented on its variance with optimal ratio (about 2:1) necessary for inhibition of excessive bone resorption in adult animals (27, 29). They stated that high dietary phosphorus in the U.S. diet warrants consideration due to the

fact it may induce a mild chronic secondary hyperparathyroidism with attendant bone resorption.

Wachman and Bernstein (33) have hypothesized that increased incidence of osteoporosis with age may represent, in part, the result of utilization of the buffering capacity of the inorganic salts of bone for maintaining pH homeostasis. Numerous studies have suggested that bone salts are dissolved in response to acid loading in normal people (34, 35, 36), and in renal acidosis (37). Barzel and Jowsey (38) and Barzel (39) have reported that chronic ingestion of ammonium chloride by adult rats resulted in significant loss of bone, including both the organic and inorganic phases of bone. The changes were independent of the calcium level of the diet and not associated with changes in blood calcium or phosphorus. The addition of sodium and potassium bicarbonate to a low calcium diet prevented the reduction of bone fat-free solid and bone calcium caused by the diet.

II. RENAL RESPONSE TO METABOLIC ACIDOSIS

Ingestion of ammonium chloride provides a satisfactory method for inducing metabolic acidosis in man and certain other mammals, since ammonium ions are rapidly converted to hydrogen ions, glutamine and urea in the liver (40). Therefore, in effect, ammonium chloride ingestion is comparable to the addition of hydrochloric acid to the body fluids (41).

The renal response in man to ammonium chloride loading has been described by numerous investigators (34-37, 42-45). The effects of metabolic acidosis produced by this agent in a normal, 60 kg subject

have been reviewed, recently, by Simpson (46). The immediate response of systemic acid-base balance to the increased acid load was a decline in plasma bicarbonate accompanied by a sharp fall in urine pH. Titratable acid and ammonium excretion rose as the kidneys compensated for the increased acid load by excreting more hydrogen ions. After 4 days the bicarbonate reached a minimum, and then rose and stabilized. Change in pH paralleled the rise in bicarbonate and titratable acid declined. Ammonium excretion, however, continued to rise for several more days before leveling off. A stable state of metabolic acidosis was achieved after about 10 days. The values for urine pH, titratable acid and ammonium excretion in the period immediately following onset of the acidosis showed a marked contrast with those subsequently obtained after a constant degree of acidosis was achieved, and the researcher emphasized the importance of studying acid excretion during a steady state of acidosis rather than during the days immediately after the hydrogen ion load is increased. Acute metabolic acidosis stimulated a prompt diuresis of sodium, potassium, calcium, and phosphorus. After a steady state acidosis developed the urinary losses of sodium, potassium and phosphorus declined but the excretion of calcium remained considerably elevated, probably reflecting continued loss of calcium from bone in exchange for hydrogen ions (46).

The initial fall in urine pH and its subsequent rise paralleled the changes in bicarbonate and represented stimulation of hydrogen ion secretion by acute metabolic acidosis (46). The changes in titratable acid reflected changes in hydrogen ions bound to buffers in the urine which occur when pH fluctuates (46). Another influence on titratable

acid excretion was a slight increase in the amount of phosphate excreted, increasing the amount of buffered hydrogen ions at any given urine pH. Phosphate excretion was maximum at about 50 percent above control values on the fourth and fifth days of acidosis then fell to a steady state level of 14 percent above control values (46).

Lemann et al. (47) reported that high concentrations of calcium and ammonium in the urine which occurred during ammonium chloride loading of normal subjects led to overestimation of titratable acid when measured by direct titration. However, they have stated that overestimation due to these factors is only about 2 percent of total renal acid excretion, in situations of induced acidosis.

The adaptive increase in urinary ammonium excretion following an acid load is characteristic of several animal species, including man (45, 48, 49), dog (50), and rat (51, 52). Some of the adaptive increase in excretion of ammonia could result from a favorable change in factors that determine rate of ammonia diffusion (41). It is now believed that ammonia enters the tubular lumen by a single diffusion process governed by the hydrogen-ion concentration gradient between cell and tubular urine, and by the proportion of the intracellular ammonia that exists as the more-lipid soluble, more permeable, un-ionized free base at the prevailing intracellular pH (53).

Another adaptive mechanism which has been observed is the increase of enzymes implicated in production of ammonia within the tubular cells (52, 54). The major source of urinary ammonia is glutamine (55). Glutamine is degraded to ammonia similarly in the dog and the

rat (50, 54). Two major pathways exist:

1. Glutamine may be deaminated to glutamic acid and ammonia (the glutaminase-I reaction) and the glutamic acid converted to α-ketoglutarate and ammonia (the glutamic dehydrogenase step). The carbon skeleton of glutamine, α-ketoglutarate, may in addition to other paths, traverse the Krebs cycle or be converted to glucose.

2. Glutamine may be transaminated with keto acids; the α-ketoglutaramate so formed then converted to α-ketoglutarate and ammonia (Glutaminase-II reaction)(56). Thus metabolic acidosis causes an adaptive change in intact cells of the renal cortex which results in an increased rate of glutamine metabolism and ammonia production. Although it has been a subject of study and speculation for a number of years, the genesis of adaptation remains obscure.

Three enzymes, which are capable of deaminating glutamine, occur in all mammalian kidneys examined (57, 58). Goldstein (54) attempted to determine the relative activities of phosphate-dependent glutaminase (PDG), phosphate-independent glutaminase (PIG), and glutamine-ketoacid aminotransferases (GKA) under conditions existing in the rat kidney. His results suggested that PDG and PIG but not GKA play a significant role in renal glutamine deamination and ammonia production in the rat kidney. The increase in renal ammonia production in rats (52, 54) and mice (59) is attributed to intensified PDG activity.

Chronic ammonium chloride acidosis has been observed to cause renal enlargement in rats (60, 61) and mice (59). In order to assess whether increased PDG activity during chronic ammonium chloride acidosis

in rats is associated with increased cell number, Janicki (61) incorporated tritiated thymidine into renal DNA and found no increase. To substantiate the observation, he injected acidotic rats with hydroxyurea, an inhibitor of DNA synthesis, and observed that PDG activity was elevated to the same extent as in acidotic rats injected with saline. These data indicated that DNA synthesis and cell division are not an important part of renal enlargement during ammonium chloride acidosis in rats. Janicki (61) suggested that the existing population of tubule cells have a remarkable capacity to adapt enzymatically to an acid insult.

III. BONE RESPONSE TO METABOLIC ACIDOSIS

When a mineral acid is administered to man or animal, a substantial fraction is buffered in sites other than blood or interstitial fluid. Originally, Van Slyke and Cullen (62) and Haldane (63) suggested that a large part of the neutralization of administered acids is done by tissue buffers. These earlier workers based their hypothesis on the observation that administration of acidifying substances resulted in the buffering of much more acid than could be accounted for by the decrease in blood bicarbonate. Later studies provided more detailed quantitative data on the internal distribution of administered hydrogen ions. Swan and Pitts (64) in nephrectomized dogs and Schwartz et al. (65) in normal human subjects have demonstrated that only about one-half of a mineral acid load is buffered in the extracellular space and red blood cells. The remaining hydrogen ions exchange with sodium and

potassium from tissue and bone and are presumably buffered in these areas.

Goodman et al. (37) demonstrated that patients with severe chronic renal acidosis excreted significantly less acid in their urine than was released endogeneously by metabolic processes. In spite of the apparent positive net balance of acid, patients maintained a steady, although low, level of plasma bicarbonate. After the acidosis had been corrected and plasma bicarbonate had been maintained at normal levels by the daily administration of sodium bicarbonate, the apparent retention of fixed acid disappeared. They concluded that severe acidosis per se calls into play some mechanism that helps to stabilize the extracellular bicarbonate level by disposing of that part of the endogenous acid load not excreted in the urine. The nature of this extrarenal mechanism was not identified, but the researchers suggested neutralization of acid by reaction with bone salts.

In the same laboratory, Lemann et al. (34) demonstrated that the same mechanism is operative even where there is no renal disease. Normal subjects given large loads of ammonium chloride were in continuously positive acid balance in spite of low but stable serum carbon dioxide content and blood pH. Sustained administration of sodium bicarbonate to normal subjects caused a continuous retention of alkali (i.e. a negative balance of acid) although there was no progressive rise in serum CO_2 content or blood pH. They suggested that slow dissolution of alkaline bone salts, stimulated by extracellular acidosis, could account for the apparent retention of chronic acid

loads and that part of the initial response to sustained acidosis probably also involves exchange of hydrogen ions with sodium and potassium bound in tissues. They observed large diuresis of calcium and phosphorus during administration of ammonium chloride, which they interpreted as lending support to the suggestion that bone would be a logical source of buffer.

Lemann et al. (35) carried out metabolic balance studies in five normal subjects to study further the relationship between acid retention and calcium balance during chronic ammonium chloride acidosis. Their results showed that initially acid was retained and appeared to titrate extra- and intracellular buffer systems. As acid retention continued, intracellular and bone buffers and finally, bone mineral alone, appeared to provide additional buffer reserves. Urinary phosphorus losses began immediately with acid loading and stopped abruptly when the load was discontinued, whereas calcium losses rose slowly and progressively during acidosis and slowly returned to normal levels after ammonium chloride loading was stopped.

Martin et al. (66) determined urinary excretion of magnesium, calcium and phosphate in 6 normal subjects during 5 days of ingestion of ammonium chloride. Their results showed statistically significant increases in urinary output of magnesium, calcium and phosphate during acid loading as compared to a 5-day control period.

To study the mechanism by which chronic metabolic acidosis augments urinary calcium excretion in man, Lemann et al. (36) carried out clearance studies in nine healthy adults and four patients with

hypoparathyroidism before and after inducing stable metabolic acidosis with either ammonium chloride or acetazolamide. During acidosis in normal subjects, the serum ultrafilterable calcium concentration rose significantly, but the clearance of inulin fell to a greater extent so that the calculated filtered load of calcium fell significantly. In spite of this, urinary calcium rose. During the recovery studies when the serum ultrafilterable calcium concentration and filtered load of calcium had returned to control levels, urinary calcium excretion remained elevated. They presented evidence indicating that the increased calcium excretion which occurred during acidosis and recovery clearances was not due to natriuresis or to increased excretion of complexing anions. Comparable results were obtained in the four patients with hypoparathyroidism, two of whom also had hypothyroidism. They suggested that the capacity to alter secretion rates of parathyroid hormone, thyrocalcitonin or both is not a critical determinant of the augmented rates of calcium excretion during acidosis. The researchers concluded that metabolic acidosis produces increased urinary calcium excretion by causing decreased renal tubular calcium reabsorption, and presented evidence that this is a direct effect of metabolic acidosis on metabolic processes within renal tubular cells.

Numerous animal experiments have shown that acidosis affects bone. Irving and Chute (67) fed rats hydrochloric acid and demonstrated a fall in bone carbonate without a change in muscle carbon dioxide. Acidosis in rats, induced by ammonium chloride, caused loss of bone sodium (68, 71) which was accompanied by a loss of bone carbonate (45).

Logan (72) demonstrated marked calciuria in rabbits made acidotic with ammonium chloride and computed that this calcium must have come from the skeleton.

Jaffee et al. (73) fed 3, 6, 8, and 18-month-old dogs calcium-low and calcium-adequate diets with and without added ammonium chloride for an 11-week period. Their results showed that in all age groups the effects of ammonium chloride administration were found to be strikingly dependent upon the calcium intake. Dogs receiving an adequate calcium diet and ammonium chloride showed less decalcification than those receiving a low calcium diet with or without ammonium chloride. In the younger groups the added effect of ammonium chloride to calcium-low diet brought out more striking changes than a low calcium diet alone. In the oldest dogs fed the low calcium diet with or without ammonium chloride, and in the younger dogs on an adequate calcium intake with ammonium chloride decalcification was less severe, as shown by generalized thinning of the bones without marrow fibrosis (osteoporosis). When decalcification was rapid and severe as in the youngest dogs fed the calcium diet, particularly with ammonium chloride, generalized decalcification and secondary marrow fibrosis (ostitis fibrosa) resulted.

Barzel and Jowsey (38) fed 250 g male Sprague-Dawley rats regular laboratory rat diet containing 800 mg calcium/100 g diet and 180 mg phosphorus/100 g diet, and a low calcium diet containing 20 mg calcium/100 g diet and 165 mg phosphorus/100 g diet, both with and without 1.5% ammonium chloride in lieu of drinking water, and the same low calcium diet with 0.25% sodium bicarbonate and 0.30% potassium bicarbonate

in their drinking water for 330 days. Their results showed that chronic ingestion of ammonium chloride by adult rats maintained on a diet adequate in vitamin D resulted in significant loss of bone tissue, including both organic and inorganic phases of bone, and that the changes were independent of the level of dietary calcium and not associated with changes in blood calcium or phosphorus. On the basis of microradiographic studies they suggested that the bone loss resulted from increased resorption. Prolonged ingestion of sodium and potassium bicarbonate prevented the bone loss in the rats fed the low-calcium diet but did not affect their blood levels of calcium or phosphorus. They postulated that cellular mechanisms involved with bone formation and bone resorption may be responsive to acid-base balance, affecting the storage of alkaline bone mineral as bone during the ingestion of excess alkali and the release of bone mineral by bone resorption during periods of acidosis.

In another study Barzel (39) fed male Sprague-Dawley rats with an average weight of 550 g the same regular laboratory diet and low calcium diet, both with and without 2.0% ammonium chloride in lieu of drinking water for a period of 6 months. Results showed significant reduction in bone density of animals fed the low calcium or ammonium chloride containing diets. Animals receiving the low calcium diet and ammonium chloride showed significant decreases in fat free solids (FFS) and bone calcium as well.

IV. AGE-RELATED CHANGES IN RENAL FUNCTION

In 1938 Lewis and Alving (74) reported that certain functions of the kidneys are altered with age. They measured urea clearance, blood urea nitrogen, and concentrating ability of the kidneys in normal men, twenty in each decade, from 40 to 89 years, two of 91 years, and one of 101 years. Their results showed that urea clearance, expressed in percent of average clearance in young normal men, declined from 100% at 40 years to 55% at 89 years. In the same men, blood urea nitrogen increased, and the concentrating ability of the kidneys, as shown by the urinary specific gravity declined.

Later, Davies and Shock (75) studied age changes in glomerular filtration rate, effective renal plasma flow, and tubular excretory capacity in adult males. Measurements of inulin clearance, diodrast clearance and diodrast Tm (maximum rate of transport of actively reabsorbed or secreted substances--the "transport maximum") were made under basal conditions in 70 males between the ages of 20 and 90 years. Nine to 12 subjects were selected from each decade on the basis of medical history, physical examination, and urine analysis. All subjects were free from history or clinical evidence of renal disease, essential hypertension, cerebrovascular accident, or heart disease. They were ambulatory and afebrile. The average inulin clearance, diodrast clearance and diodrast Tm decreased linearly beyond the age of 30 years. Filtration fraction showed a significant rise between the third and ninth decade. Inulin clearance per unit of diodrast Tm

did not change significantly. There was a small reduction in diodrast clearance per unit of diodrast Tm over the age span studied.

Because the study of Davies and Shock (75) showed decreased tubular function in the older individual, as evidenced by a decrease in maximal rate of diodrast excretion, doubt was raised as to the validity of the assumptions involved in the use of diodrast or p-amino hippurate clearance as a measure of renal plasma flow in the aged. Miller et al. (76) were led to study renal extraction of p-amino hippurate (PAH) at low plasma levels to determine whether renal plasma flow is actually reduced with age or whether impaired tubular function may be responsible in part or whole for the observed decreases in clearances. They determined the renal extraction of PAH at low plasma levels in 27 subjects who ranged in age from 23 to 75 years. They found that the fraction of PAH extracted by the kidney of the older individual was the same as the fraction extracted by the kidney of the young individual. Although the renal extraction of PAH was not age-related, the clearance of PAH was decreased in older individuals. They concluded that since the renal extraction of PAH does not change with age that the clearance of p-amino hippurate is a valid measure of renal plasma flow in the old as well as in the young, and the observed decreases in the clearance of diodrast and PAH in older individuals are caused by an actual decrease in renal plasma flow and the indiviudal tubules of the old kidney retain their ability to extract PAH completely at low plasma levels.

Since the kidney tubules regulate one aspect of the acid-base equilibrium of the blood, studies have been conducted to determine the effect of age on this renal function. Shock (77) made determinations of the acid-base balance of the blood of 40 males between the ages of 75 and 85 years. All of the aged subjects were free from clinical signs or previous history of renal or cardiovascular disease. Samples of finger blood were analyzed for pH, percentage of red cells, total CO_2 content, and pCO_2 of arterial blood, and computations for the bicarbonate content of plasma were made. Average values were determined and compared with similar determinations made on 100 young adults between the ages of 18 and 25 years. The results showed that the percentage of red cells, the pH and the bicarbonate content of the serum were significantly lower while the pCO_2 was higher in the aged subjects than in the young adults.

Shock and Yiengst (78) investigated acid-base equilibrium of blood under resting conditions in 152 males between the ages of 40 and 89 years. A significant reduction in cell volume and pH was observed with age, but changes in pCO_2, total CO_2 content of the blood and bicarbonate content of the serum were not statistically significant. They found no significant relationship between depressed renal function (glomerular filtration rate, effective renal plasma flow, and Tm diodrast) as measured in 34 of the subjects and alterations in the acid-base equilibrium of the blood under resting conditions. They suggested that the lack of significant age differences in the daily variations in acid-base equilibrium is evidence that elderly subjects

retain their homeostatic capacities with respect to this function and are able to adjust to the daily demands for adequate excretion of acid or alkali.

Although normal under conditions of rest, regulatory mechanisms for maintaining acid-base equilbrium in geriatric subjects, have been reported to show impairment under conditions of physiologic stress. Shock and Yiengst (79) induced experimental displacement in the acid-base equilibrium of the blood in six males, aged 70 to 90 years by oral administration of sodium bicarbonate or ammonium chloride. Blood samples, which were drawn at one-half to hour intervals for 9 hours after administration of the salt were analyzed for pH, percent red cells, total CO_2 content, pCO_2 and the bicarbonate content of plasma. Results on the aged were compared with those on young adults. Their results indicated that recovery from metabolic acidosis induced by the oral administration of ammonium chloride was significantly slower in the aged subjects tested than in the young adults. Eight hours after the administration of the salt, the average recovery of blood bicarbonate levels was 70 percent complete in young subjects, while in the old subjects the average recovery was only 27 percent complete. There was only a slight difference between aged males and young adults in the rate of elimination of orally ingested sodium bicarbonate.

Hilton et al. (80) investigated the effect of prolonged administration of ammonium chloride on the blood acid-base equilibrium of elderly subjects. Tests were made on a group of six males between the ages of 17 and 38 (control group) and five males and one female

between the ages of 65 and 73. All subjects were free of cardiovascular and renal disease. Ammonium chloride, in a dosage of 1.5 mEq/kg of body weight per day was administered three times a day for from four to fourteen days. Heparinized blood samples were analyzed for pH and total carbon dioxide of the plasma; plasma bicarbonate concentration was calculated. In the control group blood acid-base equilibrium studies showed complete compensation to the acid load by the fourth to seventh day (average 5.7 days), whereas blood acid-base balance remained uncompensated and a state of metabolic acidosis persisted for as long as the acid load was administered (average 12.3 days) in the experimental group. The geriatric group showed more severe metabolic acidosis induced by the standard dose of ammonium chloride than the young group.

To further clarify the effect of age on the kidney's ability to regulate systemic acid-base balance Adler et al. (81) studied the effect of acute acid loading on urinary acid excretion in 26 normal male volunteers of various ages, who were given an acute acid load consisting of 0.1 g/kg of body weight of ammonium chloride. Urinary acid excretion was followed for an eight hour period. Glomerular filtration rate and the renal blood flow were measured prior to and during ammonium chloride loading by means of inulin and PAH clearances. Their results indicated that the aged kidney's ability to excrete an acid load was impaired acutely as shown by decreased ammonia excretion and reduced total acid excretion. However, this impairment was proportional to the decrease in glomerular filtration rate. In spite

of markedly reduced glomerular filtration rate the older subjects were capable of lowering the urinary pH to at least the level seen in the younger subjects. They investigated, also, the possibility that in old subjects there could be a decrease in glutamine or a deficiency of the enzyme glutaminase. Control blood glutamine levels were measured in four young and four old subjects, who were then given ammonium chloride and glutamine to ingest. Their results showed a similar response to ingested glutamine in all subjects, suggesting that aged and young kidneys respond to amido or amino nitrogen in a nearly identical manner.

These researchers interpreted their overall results as being consistent with the hypothesis that the decrease in acid excretion found in advancing age is due to a decreased renal tubular mass rather than a specific tubular defect. They suggested that the decreased acid excretion in the elderly could be due to diminished acid production accompanied by decreased acid excretion and normal hydrogen ion balance, or that it could be a change in diurnal rhythm with increased age. Another possible explanation they suggested was that old subjects were in positive hydrogen ion balance despite normal serum bicarbonate concentrations, indicating titration of fixed buffer stores such as bone.

Laboratory rats are used frequently to study the effect of age on renal functions. Everitt (8 2) conducted a study of changes in selected renal functions throughout the life cycle of the rat. One hundred male albino rats of a non-inbred Wistar strain were used in the study. Single 24-hour specimens of urine were collected at four stages

in the life cycle: in young adult life at 240 days, in middle age at 350 days, in old age at 750 days and in senility at 950 days, and analyzed for protein, creatinine, uric acid and nonprotein nitrogen. The results showed that urinary excretion of protein in the male rat increased sharply with age, showing a tenfold increase from youth to senility. The excretion of creatinine decreased progressively with age commencing in middle age and reaching the lowest level just before death. The excretion of both uric acid and nonprotein nitrogen expressed as mg per g of food consumed increased in middle age when growth ceased. A second rise in the excretion of these constituents occurred in the last 200 days of life, as the rat approached death.

Elevated excretion of protein by old rats has been reported by Berg (83) and Perry (84), also. In agreement, Beauchene et al. (85) observed markedly higher excretions of urinary proteins in old rats (23 to 28-month-old) than in young rats (11 to 14-month-old), and reported an increasing proportion of albumin among urinary proteins with age. It has been suggested that the latter finding may be due to the increased incidence with age of glomerulonephritis in the rat (83, 85, 86).

Dicker and Nunn (87) and Radford (88) have reported decreased ability of old rats to concentrate urine. This change has been interpreted as decreased ability of tubule cells of older animals to perform osmotic work (89).

Adams and Barrows (89) used an in vitro system to study the ability of kidney tubule cells of older rats to perform osmotic work.

This renal tubular function in young, growing (2-3.5 months), adult (12-15 months), and senescent (26-33 months) female rats was estimated by the active accumulation of PAH by kidney slices. The concentrations of DNA in the slices were determined as indices of the number of cells in the tissue preparation. They observed no effect of age in PAH accumulation by kidney slices in a system containing low concentrations of PAH (3.88 µg/ml) or after 15 minutes incubation in a system containing high concentrations of PAH (200 µg/ml). However, marked agewise reductions were observed following 60 minutes of incubation. They concluded that the maximal accumulation of PAH by kidney cells in rats is markedly reduced with age, since these decrements exceeded the age-dependent decreases in the concentration of DNA.

Using the same in vitro technique Beauchene et al. (90) showed that the decrement in PAH accumulation by rat kidney slices with age was not due to net efflux of PAH during extended periods of incubation. They also showed that the ability of kidney slices to accumulate alpha-aminoisobutyric acid (AIB), a compound whose biochemical transport pathway differs from that of PAH, also decreased with age. Age-associated decrements in the sodium potassium-activated ATPase activity of renal tissue were observed, but age had no effect on the tissue potassium concentration following the accumulation of PAH.

Gregory and Barrows (91) have employed clearance techniques in rats similar to those used in man to determine the effect of age on renal function and to make valid comparisons between the two species. Adult (12 month), old (24 month) and senescent (30 month) unmated

female rats of the Wistar Strain were fitted with catheters under ether anesthesia. One catheter was inserted into the right carotid artery, a second into the right jugular vein and a third into the bladder. Following a convalescent period of about five days renal clearances were performed on the rats. A solution containing inulin and PAH was perfused into the venous catheter. Blood samples and urine collections were made at specified intervals and analyzed for PAH and inulin. Clearance rates of these substances were then calculated. There were no observed changes in glomerular filtration rate in rats between the age of 12 and 30 months, but a significant decrease (25%) was found in the Tm PAH of 30 month compared to 12 or 24 month old animals. Their data indicated that when the same testing procedure was used, the effect of age on the kidney differs in men and rats. They suggested that the degree of renal cell loss is the major difference between the species. The researchers postulated that the greater susceptibility of man than rat to vascular disease such as arteriosclerosis which results in ischemia and cell loss is probably the cause of the apparent species difference in the effect of age on the kidney.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Albino, male weanling rats and male 10-month old retired breeder rats of the NLR Strain, Wistar origin, were obtained from National Animal Laboratory Company, Creve Coeur, Missouri for this study. All animals were maintained on a commercial laboratory chow, Wayne Lab Blox, until they were transferred to the experimental diets. Wayne Lab Blox contained 24.5% protein, 4.2% fat, 3.2% fiber, 1.2% calcium, 1.0% phosphorus and other essential minerals and vitamins. The weanling rats were transferred to the experimental diets at 4 months of age; the retired breeders at 16 months of age. All of the animals were offered feed and provided with distilled water ad libitum throughout the study. The animals were housed 4 or 5 to a cage in 17" x 11" x 9" wire mesh cages. The experimental diets were semi-synthetic containing either "normal" or "low" levels of calcium with or without ammonium chloride as an acid stress. Sodium and potassium bicarbonate as a source of base were added to the diets of some of the acid-stressed rats in both age-groups and on both levels of dietary calcium. Also, some rats in each age group were placed on experimental diets in which sodium chloride was substituted for ammonium chloride. Another group of rats in each age group was continued on lab chow for the duration of the

study. Mean daily feed intakes were determined during the fourth, eight, and sixteenth weeks after the animals were placed on experimental diets, by measuring the total feed consumption and dividing by the number of rats. The rats were weighed initially and once each month during the experimental period. The rats remained on the experimental diets 9 months, that is, until the "young" animals were 13 months of age and the "old" ones 25 months of age at which time the animals were sacrificed for biochemical studies. A summary of the animal groups and their diets is shown in Table 1.

Diets

The composition of the semi-synthethic diets is shown in Table 2. The composition of the salt mixture and of the acid-base-alphacel mixture, both of which were incorporated into the experimental diets at a constant level, e.g., 3.5% and 4.5%, respectively, were varied to obtain the experimental diets. The composition of the salt mixtures (Table 3) was altered to provide 100 mg (low) and 500 mg (normal) of calcium per 100 g of diet. The calcium level of the "low calcium" salt mixture was obtained by substituting non-nutritive bulk for the $CaCO_3$ in the "normal calcium" salt mixture. These levels, as well as those of selected other mineral elements in the diet, provided by the salt mixtures are shown in Table 4. Both salt mixtures were a modification of a salt mixture (UCB-1Rb) used by Williams et al. (92).

The variable composition of the experimental diets as provided by the acid-base-alphacel mixtures is shown in Table 5. Based on preliminary testing for tolerance level, ammonium chloride was incorporated

TABLE 1

ANIMAL GROUPS AND OVERALL DIETARY TREATMENT

Group	Number	Diet Code	Calcium mg/100 g	Acid[1] Added	Base[2] Added
Young (Initial age 4 months)					
YCHOW	12	CHOW	1200	-	-
YN	12	N	500	-	-
YNA	12	NA	500	+	-
YNAB	12	NAB	500	+	+
YNBSALT	12	NBSALT[3]	500	-	+
YL	12	L	100	-	-
YLA	12	LA	100	+	-
YLAB	12	LAB	100	+	+
Old (Initial age 16 months)					
OCHOW	15	CHOW	1200	-	-
ON	15	N	500	-	-
ONA	15	NA	500	+	-
ONAB	15	NAB	500	+	+
ONBSALT	10	NBSALT[3]	500	-	+
OL	15	L	100	-	-
OLA	15	LA	100	+	-
OLAB	15	LAB	100	+	+

[1] 2.0 g NH_4Cl per 100 g of diet.

[2] 0.33 g $NaHCO_3$ and 0.40 g $KHCO_3$ per 100 g of diet.

[3] 2.18 g NaCl per 100 g of diet.

TABLE 2

COMPOSITION OF EXPERIMENTAL DIETS

Component	Percent of Diet
Casein[1]	20.00
Cornstarch	30.00
Sucrose	30.00
Crisco	8.00
Wesson Oil	2.00
Vitamin Mixture[1]	2.00
Salt Mixture	3.50
Acid-Base-"Alphacel"[1] Mixture	4.50

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 3

COMPOSITION OF SALT MIXTURES[1]

Component	Percent of Salt Mixture	
	Normal Calcium	Low Calcium
"Alphacel"[2] non-nutritive bulk	-	28.56
$CaCO_3$	28.56	-
Na_2HPO_4	27.56	27.56
K_2HPO_4	17.86	17.86
$CaHPO_4$	9.72	9.72
KCl	8.76	8.76
$MgSO_4$	6.57	6.57
$MnSO_4 \cdot H_2O$	0.4400	0.4400
$FeC_6H_5O_7 \cdot 5H_2O$	0.4310	0.4310
$ZnCO_3$	0.0600	0.0600
$CuSO_4$	0.370	0.0370
KIO_3	0.0370	0.0030
Na_2SeO_4	0.0003	0.0003

[1] 3.5% of diet.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 4

LEVELS OF MINERALS IN THE DIET COMPARED WITH LEVELS REQUIRED FOR GROWTH OF RATS

Mineral Element	Required for Growth[1]	Per 100 g of Diet						
		N	NA	NAB	NBSALT	L	LA	LAB
Calcium, mg	560.0	500.0	500.0	500.0	500.0	100.0	100.0	100.0
Phosphorus, mg	440.0	400.0	400.0	400.0	400.0	400.0	400.0	400.0
Sodium, mg	60.0	313.0	313.0	403.0	1260.0	313.0	313.0	403.0
Potassium, mg	200.0	440.0	440.0	596.0	596.0	440.0	440.0	596.0
Chlorine, mg	60.0	146.0	1470.0	1470.0	1470.0	146.0	1470.0	1470.0
Magnesium, mg	40.0	46.0	46.0	46.0	46.0	46.0	46.0	46.0
Manganese, mg	5.6	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Iron, mg	3.9	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Copper, mg	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Zinc, mg	1.3	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Iodine, mcg	17.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Selenium, mcg	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0

[1]NRC Committee on Animal Nutrition (93).

TABLE 5

VARIABLE COMPOSITION OF THE EXPERIMENTAL DIETS AS PROVIDED BY THE ACID-BASE MIXTURE[1]

Diet		Ammonium Chloride	Bicarbonate		Sodium Chloride	Non-nutritive Bulk
Name	Code	Percent	Sodium Percent	Potassium Percent	Percent	Percent
Normal Calcium	N	-	-	-	-	4.50
Normal Calcium + Acid	NA	2.00	-	-	-	2.50
Normal Calcium + Acid + Base	NAB	2.00	0.33	0.40	-	1.77
Normal Calcium + Salt + Base	NBSALT	-	0.33	0.40	2.18	1.59
Low Calcium	L	-	-	-	-	4.50
Low Calcium + Acid	LA	2.00	-	-	-	2.50
Low Calcium + Acid + Base	LAB	2.00	0.33	0.40	-	1.77

[1] 4.5% of diet.

into the "acid" containing diets at the level of 2.0%; sodium bicarbonate and potassium bicarbonate were incorporated into the "base" containing diets at the level of 0.33% and 0.40%, respectively. In the diets of 2 groups of rats, one of each age, sodium chloride, at the level of 2.18% replaced ammonium chloride in order to ascertain the effect of osmotic "load" on the observed renal changes.

II. METHODS

Urine Collection

After young and old rats had been fed the experimental diets for a period of 6 to 8 months, they were placed in metabolism cages and two 72-hour urine collections were obtained from each rat.

Daily 24-hour urine samples from each rat were collected in 1 ounce acid-rinsed bottles using 1 drop of mineral oil (52) and 0.2 ml of a 10% (w/v) solution of thymol in propanol as a preservative (94). At the end of each 24-hour period, the urine in each collection bottle was transferred to an acid-rinsed centrifuge tube and centrifuged[a] at 1500 x g for 5 minutes to remove feed particles from the urine sample. Then the urine was decanted into an acid-rinsed plastic bottle, covered and refrigerated until the 72-hour urine collection was completed. Each day freshly prepared collection bottles were provided and the funnels of the metabolism cages were rinsed with water. At the end of the three-day

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[a]International Centrifuge Model SBB #50092 H.

collection period, the pooled 24-hour urine samples were transferred to acid-rinsed graduated cylinders and the 72-hour urine volume was determined.

Determination of Urinary pH and Titratable Acid

Urinary pH and titratable acid (TA) were determined with an Arthur H. Thomas combination microelectrode and a Sargent-Welch pH meter. TA was determined using a modification of the method described by Goldstein (95). In this method, an aliquot of urine is titrated to pH 7.4 with standard alkali.

Reagent.

Sodium Hydroxide: approximately 0.01 N, standardized.

Procedure. One ml of each 72-hour urine sample was pipetted into a 15 ml beaker which was positioned on a magnetic stirrer and the original pH was determined. Then the urine sample was titrated with constant stirring to pH 7.4 with the standardized NaOH using a 10 ml automatic-fill microburette. Before each series of titrations, the N of the standardized base was checked to assure uniform strength of the reagent. TA excretion, μequivalents per 100 g body weight per hour was calculated as follows:

$$\text{TA, } \mu\text{Eq/100 g Body Weight/Hour} = \frac{\text{ml Urine/Hour} \times \text{ml NaOH} \times \text{N NaOH} \times 1000}{\text{Body Weight in 100 g Units}}$$

Determination of Urinary Ammonia Nitrogen

The ammonia nitrogen content of the urine samples was determined in duplicate using the Berthelot color reaction (96, 97, 98). The Berthelot

reaction takes place between ammonia and phenol in the presence of hypochlorite and sodium nitroprusside, nitrogen thus being converted to indophenol. The blue color of the end product can be measured spectrophotometrically. Preliminary determinations of ammonia nitrogen in urine samples obtained from young and old rats, which were fed the various experimental diets, using the Berthelot color reaction with and without microdiffusion showed no significant differences. Therefore, the microdiffusion step was omitted from the determination.

Reagents.

1. Ammonia Nitrogen Standard: 0.0472 g of ammonium sulfate was dissolved in water and diluted to 1 liter. One ml of the standard contained 10 µg of ammonia nitrogen. Working standards containing 0 to 8 µg ammonia nitrogen per 1 ml solution were prepared for each series of determinations.

2. Phenol Color Reagent: 60 g of phenol (A. R. grade[and 0.25 g of sodium nitroprusside (A. R. grade) were dissolved in water. The mixture was heated over a steam bath until the phenol went into solution, cooled and made up to 1 liter.

3. Alkali-Hypochlorite Reagent: 25 g NaOH was dissolved in 40 ml commercial bleach (Clorox) and diluted to 1 liter with water.

Procedure. Urine samples from rats, which were fed diets containing normal calcium (N), low calcium (L), normal calcium with added sodium chloride, sodium bicarbonate and potassium bicarbonate (NBSALT) or lab chow (CHOW) were diluted 1:250. Urine samples from rats which were fed

normal calcium or low calcium diets plus ammonium chloride (NA, LA) and normal calcium or low calcium diets plus ammonium chloride, sodium bicarbonate and potassium bicarbonate (NAB, LAB) were diluted 1:1000.

To 1 ml of each standard, and to 1 ml of dilute urine from rats which were fed N, NBSALT, and CHOW diets, and to 0.50 ml of diluted urine from rats which were fed L, NA, NAB, LA, LAB diets were added 0.5 ml phenol color reagent and 0.5 ml alkali-hypochlorite reagent. The contents of all of the tubes were diluted to 5 ml with water and mixed on a mechanical mixer. After incubation for 30 minutes in a 37° water bath, the absorbances (A) of the samples were determined at 540 mm using a Beckman B spectrophotometer. The µg ammonia nitrogen per hour were calculated as follows:

$$NH_3\text{-N, µg/ml of Urine} = \frac{\text{A of Sample x Conc of Std x Dilution Factor}}{\text{A of Std}}$$

$$NH_3\text{-N, µg/Hour} = NH_3\text{-N, µg/ml of Urine x ml Urine/Hour}$$

$$NH_3\text{-N, µM/Hour} = \frac{NH_3\text{-N, µg/Hour}}{14}$$

$$NH_3\text{-N, µM/100 g Body Weight/Hour} = \frac{NH_3\text{-N, µM/Hour}}{\text{Body Weight in 100 g Units}}$$

Ashing of Urine Samples and Preparation of Ash Solutions

Aliquots of 72-hour urine samples obtained from all rats were dried, ashed and solutions of the ash prepared.

Reagent.

Hydrochlorice Acid, approximately 6 N: 498 ml concentrated HCl was diluted to 1 liter with water.

Procedure. Five ml aliquots of 72-hour urine samples were pipetted into labeled Coors, Size 00 crucibles. Samples were charred overnight under heat lamps, then transferred to a cold muffle furnace for ashing. Samples were ashed at 550° for a 24-hour period. The ash was taken up in 1 ml 6 N HCl and evaporated to almost dryness on a hot plate. Then, the ash was dissolved in water and the solution filtered into an acid-rinsed 10 ml volumetric flask. The contents of the flask were diluted to volume, transferred to nylon screw-top vials and stored at 0° until analyzed.

Determination of Urinary Calcium

Urinary calcium was determined on duplicate samples using a Perkin-Elmer, Model 303, atomic absorption spectrophotometer, and a modification of the method described in the manual[a] was followed. Calcium compounds when introduced into a flame dissociate into free calcium atoms. In this form calcium absorbs light of characteristic wavelength produced by a hollow cathode lamp. Under the described conditions only a small fraction of calcium atoms will be raised to a higher energy level and will emit light on returning to the ground state. Phosphate interference is eliminated by the addition of lanthanum, which

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[a]Supplement to analytical methods for atomic absorption spectrophotometry (1966) The Perkin-Elmer Corporation, Ca 8.

binds preferentially with phosphate and prevents the formation of calcium phosphates (99).

Reagents.

1. Stock Calcium Solution: 0.3121 g $CaCO_3$ was dissolved in water with the aid of 20 drops of concentrated HCl and diluted to 250 ml. This solution contained 500 mg of calcium per liter.

2. Dilute Calcium Solution: 10 ml of stock calcium solution were diluted to 100 ml. This solution contained 0.05 mg calcium per ml.

3. Lanthanum Solution 5%: 5.9 g La_2O_3 and 25 ml concentrated HCl were diluted to 100 ml with water.

4. Working standards containing 0.00 to 0.10 mg per 10 ml solution and 1 ml of 5% lanthanum were freshly prepared for each series of determinations.

Procedure. One ml of urine ash solution from rats which were fed N, L, NBSALT and CHOW diets was pipetted into acid-rinsed 16 x 150 mm test tubes followed by 8 ml of water. Five-tenths ml of urine ash solutions from rats, which were fed NA, NAB, LA and LAB diets was pipetted into like test tubes and 8.5 ml water were added. Shortly before the calcium determination, 1 ml of 5% lanthanum solution was added to each sample, and the samples were mixed using a mechanical mixer. Percent absorption was determined at 421.2 nm using a Perkin-Elmer 303 atomic absorption spectrophotometer. These values were converted to absorbances, using the Perkin-Elmer conversion table[a] and then converted to

[a]Supplement to analytical methods for atomic absorption spectrophotometry (1966), The Perkin-Elmer Corporation, p. 10.

mg calcium per 10 ml solution by referring them to the standard curve. Milligrams urinary calcium per 24 hours were calculated as follows:

$$\text{Calcium, mg/24 Hours} = \text{mg/10 ml Solution} \times \frac{\text{Dilution}}{\text{Factor}} \times \frac{\text{24-Hour}}{\text{Urine Volume}}$$

Determination of Urinary Phosphorus

Urinary phosphorus was determined on duplicate samples using a modification of the method described by Dryer et al. (100). Treatment of a phosphorus-containing solution with ammonium molybdate converts inorganic phosphorus to phosphomolybdate. Phosphomolybdate is reduced with N-phenyl-p-phenylenediamine monohydrochloride and the blue color of the end product is measured spectrophotometrically.

Reagents.

1. Phosphorus Stock Standard: 0.4381 g KH_2PO_4 was dissolved in water and diluted to 200 ml. This solution contained 0.05 mg phosphorus per ml.

2. Phosphorus Working Standards: 1, 2, 3, 4 and 5 ml phosphorus stock standard were diluted to 250 ml with water. These working standards provided 2, 4, 6, 8 and 10 µg phosphorus per ml, respectively.

3. Sulfuric Acid, 5 N: 138 ml concentrated H_2SO_4 was diluted to 1 liter with water.

4. Ammonium Molybdate, 0.025 M: 30 g $(NH_4)_6MO_7O_{24}\cdot 4H_2O$ was dissolved in water and diluted to 1 liter. One ml of concentrated H_2SO_4 was added to aid in dissolving the ammonium molybdate.

5. N-Phenyl-p-phenylenediamine Monohydrochloride, Sodium Bisulfite: Five-tenths g of N-phenyl-p-phenylenediamine monohydrochloride was moistened with a few drops of 95% ethanol in a liter flask and the contents diluted to volume with a 1% (w/v) aqueous solution of sodium bisulfite. After shaking, the solution was filtered to remove any insoluble residue.

Procedure. Urine ash solutions from all rats were diluted 1:100 before each series of determinations. To 0.4 ml dilute urine ash solution and 0.6 ml distilled water or to 1 ml of working standard, 1.0 ml 5 N H_2SO_4, 1.0 ml 0.025 M ammonium molybdate and 2.0 ml N-phenyl-p-phenylenediamine monohydrochloride solution were added. After the addition of the color reagents, the tubes were mixed on a mechanical mixer and allowed to stand for 30 minutes for color development. Absorbances (A) were determined against a reagent blank at 700 nm using a Beckman B Spectrophotometer. The mg of urinary phosphorus per 24 hours were calculated as follows:

$$\text{Phosphorus, } \mu g/ml \text{ Urine} = \frac{\text{A of sample x Conc of Std x Dilution Factor}}{\text{A of Std}}$$

$$\text{Phosphorus, mg/24 Hours} = \frac{\text{Phosphorus, } \mu g/ml \text{ Urine x 24-Hour Urine Volume}}{1000}$$

Sacrifice of Animals

After young and old rats had been fed the experimental diets for a period of approximately 9 months, randomly selected animals in each group were sacrificed for biochemical studies. All animals were allowed to eat ad libitum the night prior to sacrifice. The following morning

randomly selected animals from each age group were decapitated and blood samples were collected for calcium and phosphorus determinations. The samples were centrifuged[a] for 15 minutes at 1000 x g. The serum was collected, placed in stoppered three-inch test tubes, and maintained at 0° until assayed.

Determination of Phosphate-Dependent Glutaminase

Phosphate-dependent glutaminase activity was determined by a modification of the microdiffusion-colorimetric technique described by Janicki (61). Supernatant fluid from a kidney homogenate was incubated in a microdiffusion bottle with and without phosphate buffer and using L-glutamine as substrate. The microdiffusion technique described by Seligson (101) was used to isolate the ammonia which was formed during incubation. At the pH of the incubation mixture, 8.0, ammonia was present as ammonium ion (NH_4^+), but as free ammonia (NH_3) after the addition of potassium carbonate, pH 10.4. In this technique, ammonia was freed from the sample by the addition of potassium carbonate. The ammonia gas diffused from the sample and was absorbed on an acid-treated glass rod as ammonium sulfate; the diffusion was facilitated by rotation of the bottle in the specially constructed rotation apparatus. When microdiffusion was complete, the ammonium sulfate was rinsed from the glass rod. Berthelot color reagents were added and the ammonia nitrogen was measured spectrophotometrically. Phosphate-dependent glutaminase activity represented the difference between the amount of ammonia produced

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[a]International Centrifuge Model SBB #50092 H.

in the presence and absence of phosphate buffer, and was calculated as micromoles ammonia-nitrogen liberated per mg protein per hour (61).

Reagents.

1. Sucrose, 0.25 M: 85.6 g sucrose was dissolved in water and diluted to 1 liter.

2. Trizma Buffer, 0.05 M: 0.05 M Trizma buffer[a] was adjusted to pH 8.0 with 0.25 N NaOH.

3. Sodium Phosphate Buffer, 0.2 M: 2.4850 g Na_2HPO_4 and 0.3422 g $NaH_2PO_4 \cdot H_2O$ were each dissolved in water and each diluted to 100 ml. Then the two solutions were combined and the pH adjusted to 8.0 with 0.25 N NaOH.

4. L-Glutamine, 0.1 M: 0.3654 g L-glutamine[a] was dissolved in water and diluted to 25 ml. This reagent was prepared fresh just before each series of determinations, and it was kept on ice until used in the determinations.

5. Potassium Carbonate Solution, 45%: 450 g K_2CO_3 were dissolved in water and diluted to 1 liter.

6. Sulfuric Acid, 5 N: 138 ml concentrated H_2SO_4 was diluted to 1 liter with water.

7. Ammonia Nitrogen Standard: 0.0472 g of ammonium sulfate was dissolved in water and diluted to 1 liter. One ml of the standard contained 10 µg of ammonia-nitrogen. Working standards containing 0 to

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[a]Sigma Chemical Company, Saint Louis, Missouri.

8 µg ammonia-nitrogen per 1 ml solution were prepared for each series of determinations.

8. Phenol Color Reagent: 60 g of phenol (A. R. grade) and 0.25 g of sodium nitroprusside (A. R. grade) were dissolved in water. The mixture was heated over a steam bath until the phenol dissolved, then cooled and diluted to 1 liter.

9. Alkali-Hypochlorite Reagent: 25 g NaOH was dissolved in 40 ml commercial bleach (Clorox) and diluted to 1 liter with water.

Procedure. Following decapitation of the rats and collection of the blood samples, the kidneys were immediately removed, decapsulated, weighed and the left kidney of each animal was homogenized in ice-cold 0.25 M sucrose with a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were diluted to a final concentration of 5 percent with 0.25 M sucrose. The kidney homogenates were centrifuged[a] for 10 minutes at 600 x g at 0-2°. The reaction was carried out in duplicate in 20 ml ammonia microdiffusion bottles. The incubation mixture contained 0.2 ml Trizma buffer, 0.5 ml phosphate buffer and 0.15 ml water. Following the addition of 50 µl of the supernatant fluid, the bottles were incubated for 10 minutes in a shaking incubator at 37°. At 20 second intervals, the bottles were removed from the incubator and 100 µl L-glutamine were added to initiate the reaction. Then, they were returned to the incubator and

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[a]Lourdes Beta-Fuge Model A: 9 RA Rotor.

incubated with constant shaking for 10 minutes. Simultaneously, duplicate assays were run in which the phosphate buffer was replaced with distilled water. At the end of the second incubation period, the samples were removed from the incubator and a stainless steel mixing bar was inserted in each bottle to facilitate mixing. The reaction was terminated by adding 1 ml of 45% K_2CO_3 from a syringe, and each bottle was quickly sealed with a rubber stopper equipped with a glass rod dipped in 5 N H_2SO_4. Then, the samples were positioned in a 16-place Seligson Ammonia Diffusion Unit and diffused for 30 minutes. At the end of the diffusion period, the stoppers and glass rods were removed carefully from the bottles and the ammonium sulfate transferred into 6 ml of distilled water contained in Bausch-Lomb colorimeter tubes. Ammonia nitrogen standards containing 0 to 8 µg ammonia nitrogen per 7 ml solution were treated, similarly. The Berthelot color reagents, 0.5 ml phenol color reagent and 0.5 ml alkali-hypochlorite reagent, were added to all samples and standards. Then, the tubes were mixed on a mechanical mixer, incubated for 30 minutes in a 37° water bath and the absorbances of standards and samples with and without phosphate buffer were determined at 540 nm using a Beckman B Spectrophotometer. The ammonia produced in the diffusion bottles without phosphate buffer was subtracted from the amount produced in the presence of phosphate buffer to determine activity due to phosphate-dependent glutaminase.

Determination of Kidney Protein

Kidney protein was isolated by a modification of the method of Shibko et al. (103) and duplicate samples were assayed with the Biuret

reagent (104). The Biuret reaction results in the formation of complex ions between copper and proteins in an alkaline solution to produce a violet color, which can be measured spectrophotometrically.

Reagents.

1. Perchloric Acid Solution (PCA), 0.6% (w/v): 5 ml of 70% (w/w) perchloric acid was diluted to 1 liter with 95% alcohol.

2. Bovine Serum Albumin Standard: 1 g bovine serum albumin (BSA) was dissolved in 0.2 N NaOH and diluted to 100 ml with the same. Working standards containing 0 to 10 mg BSA per ml were prepared for each series of determinations by diluting stock standard with 0.2 N NaOH.

3. Biuret Reagent: 3 g $CuSO_4 \cdot 5H_2O$, 9 g $NaKC_4H_4O_6 \cdot 4H_2O$, 8 g NaOH and 5 g KI were dissolved in water and diluted to 1 liter.

Procedure. One-tenth ml or three-tenths ml 70% PCA were added to 1 ml of homogenate or 3 ml supernatant fluid, respectively, mixed thoroughly and chilled on ice for 15 minutes. Then, the samples were centrifuged[a] at 600 x g for 5 minutes. The supernatant fluid was decanted and the precipitate washed with 3 ml of 0.6% PCA. The samples were centrifuged again and the precipitate washed with 3 ml ether. After the third centrifugation, the supernatant fluid was discarded, and the protein pellet was dissolved in 3 ml of 0.2 N NaOH. The samples were mixed using a mechanical mixer after each addition. The samples were stoppered and allowed to stand overnight to dissolve the protein

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[a]International Centrifuge, Model SBB #50092 H.

pellet completely. To 1 ml of the protein solution, 4 ml of Biuret reagent were added. Then, the samples were mixed using a mechanical mixer, incubated for 30 minutes in a 50° water bath, allowed to cool, and the absorbances (A) were determined at 550 nm using a Beckman B Spectrophotometer. Protein, mg/100 mg renal tissue was calculated as follows:

$$\text{Protein, mg/ml NaOH} = \frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}$$

$$\text{Protein, mg/100 mg Tissue} = \text{Mg Protein/ml Homogenate x 6}$$

Phosphate-dependent glutaminase activity was calculated as micromoles NH_3 per mg protein per hour as follows:

$$\text{Protein, mg/ml Supernatant Fluid} = \frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}$$

$$\text{Protein, mg/50 µl Supernatant Fluid} = \frac{\text{Protein, mg/ml Supernatant Fluid}}{20}$$

$$NH_3\text{-N, µ M Liberated/mg Protein/Hour} = \frac{NH_3\text{-N, µg Liberated/10 minutes x 6}}{14 \text{ x mg Protein/50 µl Supernatant Fluid}}$$

Kidney weight was expressed as percent of body weight and calculated as follows:

$$\text{Percent Kidney Weight} = \frac{\text{Weight of Kidney Tissue x 100}}{\text{Body Weight}}$$

Serum Calcium Determination

Serum calcium was determined on duplicate samples using a Perkin-Elmer, Model 303, atomic absorption spectrophotometer, and a

modification of the method described in the manual[a] was followed. Proteins were precipitated by trichloroacetic acid as described by Dryer et al. (100) to eliminate the depressing effect of proteins on the measurement and to increase reproducibility and accuracy.

Reagents.

1. Trichloroacetic Acid (TCA), 100%: To one pound trichloroacetic acid in the original shipping bottle, 100 to 125 ml water were added. The mixture was placed in a 37° water bath until the crystals were dissolved. It was then transferred to a 500 ml graduated cylinder and diluted to 453.6 ml with water. The reagent was stored in the original reagent bottle at refrigerator temperature.

2. Trichloroacetic Acid (TCA), 10%: 25 ml of 100% TCA were diluted to 250 ml with water.

3. Stock Calcium Solution: 0.3121 g $CaCO_3$ was dissolved in water with the aid of 20 drops of concentrated HCl and diluted to 250 ml. This solution contained 500 mg of calcium per liter.

4. Dilute Calcium Solution: 10 ml of stock calcium solution were diluted to 100 ml. This solution contained 0.05 mg calcium per ml.

5. Stock Lanthanum Solution, 5%: 5.9 g La_2O_3 and 25 ml concentrated HCl were diluted to 100 ml with water.

6. Dilute Lanthanum Solution: 10 ml stock lanthanum solution were diluted to 50 ml with water.

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[a]Supplement to analytical methods for atomic absorption spectrophotometry (1966), The Perkin-Elmer Corporation, Ca 8.

Procedure. Three-tenths ml of each serum sample was added to 4.5 ml 10% TCA in acid-rinsed centrifuge tubes, allowed to stand 10 minutes and centrifuged[a] at 1000 x g for 10 minutes. Two ml of the clear supernatant fluid were removed and transferred to 16 x 150 mm acid-rinsed test tubes. Shortly before the calcium determinations 2 ml dilute lanthanum solution were added and the samples were mixed, using a mechanical mixer. Duplicate samples of a reference serum, Versatol[b], were treated, similarly. The percent absorptions for duplicate samples and Versatol were determined at 421.2 nm, using a Perkin-Elmer 303 atomic absorption spectrophotometer. These values were converted to absorbances (A), using the Perkin-Elmer conversion table[c]. Calcium, mg per 100 ml serum, was calculated, using the reference serum, Versatol, as follows:

$$\text{Calcium, mg per 100 ml Serum} = \frac{\text{A of Sample x Conc of Versatol}}{\text{A of Versatol}}$$

Serum Phosphorus Determination

Serum phosphorus was determined on duplicate samples by the method described by Dryer et al. (100). The serum sample was deproteinized with 10% TCA. The clear supernatant fluid was treated with ammonium molybdate which converted inorganic phosphorus to phosphomolybdate.

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[a]International Centrifuge, Model SBB #50092 H.

[b]Scientific Products, Evanston, Illinois.

[c]Supplement to analytical methods for atomic absorption spectrophotometry (1966), The Perkin-Elmer Corporation, p. 10.

Phosphomolybdate was reduced with N-phenyl-p-phenylenediamine monohydrochloride, and the resulting blue color was measured spectrophotometrically.

Reagents.

1. Trichloroacetic Acid (TCA), 10%: 25 ml of 100% TCA were diluted to 250 ml with water.

2. Phosphorus Stock Standard: 0.4381 g of KH_2PO_4 was dissolved in 10% TCA and diluted to 100 ml.

3. Phosphorus Working Standard: 1 ml of phosphorus stock standard was diluted to 100 ml with 10% TCA. Working standards, which contained 0 to 10 µg phosphorus per 3.2 ml solution, were prepared for each series of determinations.

4. Ammonium Molybdate, 0.008 M: 9.9 g $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ were dissolved in water and diluted to 1 liter. One ml of concentrated H_2SO_4 was added to aid in dissolving the ammonium molybdate.

5. N-Phenyl-p-phenlenediamine Monohydrochloride, Sodium Bisulfite: Five-tenth g of N-phenyl-p-phenylenediamine monohydrochloride was moistened with a few drops of 95% ethanol in a 1 liter flask and the contents made up to volume with a 1% (w/v) aqueous solution of sodium bisulfite. After shaking, the solution was filtered to remove any insoluble residue.

Procedure. Two-tenths ml of each serum sample was added to 1.8 ml 10% TCA in centrifuge tubes, allowed to stand 10 minutes, and

centrifuged[a] at 1000 x g for 10 minutes. One ml of the clear supernatant fluid was removed and transferred to Bausch-Lomb colorimeter tubes. Two-tenths ml ammonium molybdate and 2 ml of N-phenyl-phenylenediamine monohydrochloride were added. Then the samples were mixed on a mechanical mixer and allowed to stand 30 minutes for color development. Standards containing 0 to 10 µg phosphorus per ml were prepared, similarly. Absorbances (A) were determined at 700 nm using a Beckman B Spectrophotometer. Phosphorus, mg/100 ml serum were calculated as follows:

$$\text{Phosphorus, mg/100 ml Serum} = \frac{\text{A of Sample x Conc of Std x 1000}}{\text{A of Std}}$$

Removal and Fat Extraction of Tibias

After sacrifice of the rats, blood collection and removal of the kidneys both hind legs of the animals were severed at the head of the femur, and placed in plastic bags and stored at 0° until assayed. Later, the right tibia was removed, cleaned of adherent soft tissue, weighed and dried at 105° for 8 hours in aluminum vessels and then reweighed.

Fat extraction of the tibias was carried out in a Soxhlet extractor using a modification of the method described by Barzel et al. (38). Each tibia was wrapped in a cotton gauze square along with a small cardboard square, which had the number of the tibia marked on it with a lead

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[a]International Centrifuge, Model SBB #50092 H.

pencil and secured with thread. Eight tibias were extracted in a Soxhlet extractor at a time. The extraction solvent was a 2:1 (v/v) mixture of chloroform and methanol. After extraction, the tibias were unwrapped, air dried by exposure to air overnight and then dried at 105° for 6 hours. Following the drying process, each tibia was weighed and the fat content was determined by the difference between the dry tibia weight and the fat extracted dry weight. Percent fat of the tibia was calculated as follows:

$$\text{Percent Fat} = \frac{\text{Fat Weight of Tibia} \times 100}{\text{Dry Tibia Weight}}$$

Determination of Tibia Ash and Preparation of Ash Solutions

Tibia ash was determined using a modification of the method described by Barzel et al. (38). Each tibia was placed in a labelled Coors, Size 00 crucible, which had been brought previously to a constant weight at ashing temperature, and ashed in a muffle furnace at 550° for a 24-hour period. After ashing, each tibia was allowed to cool in a dessicator before weighing. Percent of ash of tibia was calcualted as follows:

$$\text{Percent Ash} = \frac{\text{Ash Weight of Tibia} \times 100}{\text{Fat Free Dry Tibia Weight}}$$

The ash of tibia was taken up in 2 ml 6 N HCl and evaporated to almost dryness on a hot plate. Then, the ash was dissolved in water and the solution filtered into an acid-rinsed 25 ml volumetric flask. The

contents of the flask were diluted to volume, transferred to nylon screw-top vials and stored at 0° until analyzed.

Determination of Tibia Ash Calcium

Tibia ash calcium was determined on duplicate samples using a Perkin-Elmer, Model 303, atomic absorption spectrophotometer, and a modification of the method described in the manual[a] was followed.

Reagents.

1. Stock Calcium Solution: 0.3121 g $CaCO_3$ was dissolved in water with the aid of 20 drops of concentrated HCl and diluted to 250 ml. This solution contained 500 mg of calcium per liter.

2. Dilute Calcium Solution: 10 ml of stock calcium solution were diluted to 100 ml. This solution contained 0.05 mg calcium per ml.

3. Lanthanum Solution, 5%: 5.9 g La_2O_3 and 25 ml concentrated HCl were diluted to 100 ml with water.

4. Working standards containing 0.00 to 0.10 mg per 10 ml solution and 1 ml of 5% lanthanum were freshly prepared for each series of determinations.

Procedure. Tibia ash solutions from all rats were diluted 1:100 before each series of determinations. One ml dilute ash solution was pipetted into acid-rinsed 16 x 150 mm test tubes and 8 ml distilled

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[a]Supplement to analytical methods for atomic absorption spectrophotometry (1966), The Perkin-Elmer Corporation, Ca 8.

water were added. Shortly before the determinations were to be run, 1 ml of 5% La_2O_3 solution was added to each sample, and the samples were mixed using a mechanical mixer. The percent absorptions for duplicate samples and standards were detetermined at 421.2 nm, using a Perkin-Elmer 303 atomic absorption spectrophotometer. These values were converted to absorbances (A), using the Perkin-Elmer conversion table[a] and then converted to mg calcium per 10 ml solution by referring the absorbances to the standard curve.

Percent calcium of each tibia was calculated as follows:

$$\text{Calcium, mg/ml} = \frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}$$

$$\text{Calcium, mg/Tibia} = \text{Calcium, mg/ml x 2500}$$

$$\text{Percent Calcium} = \frac{\text{Calcium, mg/Tibia x 100}}{\text{Fat Free Dry Weight of Tibia}}$$

Determination of Tibia Ash Phosphorus

Tibia ash phosphorus was determined on duplicate samples using a modification of the method described by Dryer et al. (102).

Reagents.

1. Phosphorus Stock Standard: 0.4381 g KH_2PO_4 was dissolved in water and diluted to 200 ml. This solution contained 0.05 mg phosphorus per ml.

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[a]Supplement to analytical methods for atomic absorption spectrophotometry (1966), The Perkin-Elmer Corporation, p. 10.

2. Phosphorus Working Standards: 1, 2, 3, 4, 5 and 7 ml phosphorus stock standard were diluted to 250 ml with water. These working standards provided 2, 4, 6, 8, 10 and 14 µg phosphorus per ml, respectively.

3. Sulfuric Acid, 5 N: 138 ml concentrated H_2SO_4 were diluted to 1 liter with water.

4. Ammonium Molybdate, 0.025 M: 30 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were dissolved in water and diluted to 1 liter. One ml of concentrated H_2SO_4 was added to aid in dissolving the ammonium molybdate.

5. N-Phenyl-p-phenylenediamine Monohydrochloride, Sodium Bisulfite: Five-tenths g of N-phenyl-p-phenylenediamine monohydrochloride was moistened with a few drops of 95% ethanol in a 1 liter flask and the contents diluted to volume with 1% (w/v) aqueous solution of sodium bisulfite. After shaking, the solution was filtered to remove any insoluble residue.

Procedure. Tibia ash solutions from all rats were diluted 1:100 before each series of determinations. To 0.4 ml dilute ash solution and 0.6 ml distilled water or to 1 ml of working standard, 1.0 ml 5 N H_2SO_4, 1.0 ml 0.025 M ammonium molybdate and 2.0 ml N-phenyl-p-phenylenediamine monohydrochloride solution were added. After the addition of the color reagents, the tubes were mixed on a mechanical mixer and allowed to stand for 30 minutes for color development. Absorbances (A) were determined against a reagent blank at 700 nm using a Beckman B Spectrophotometer. Percent phosphorus of each tibia was calculated as follows:

$$\text{Phosphorus, mg/0.4 ml dil ash soln} = \frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}$$

$$\text{Phosphorus, mg/Tibia} = \text{Phosphorus, mg/0.4 ml dil ash soln, x } 6.25$$

$$\text{Percent Phosphorus} = \frac{\text{Phosphorus, mg/Tibia x } 100}{\text{Fat Free Dry Weight of Tibia}}$$

Statistical Methods

The data collected were analyzed by standard statistical procedures (105). Analysis of variance was performed on body weight, feed intake, and urinary, kidney, blood and bone values. Duncan's Multiple Range Test was used to test the significance of the differences among means, which were shown to be significant by the F-test. Correlations among all variables, except feed intake were determined.

CHAPTER IV

RESULTS

I. FEED INTAKES, BODY WEIGHTS AND WEIGHT GAINS

Rats on the various diets exhibited no adverse physical symptoms during the experimental period. The analysis of variance, which was performed on the mean daily feed intake per rat fed the CHOW and experimental diets during the fourth-, eighth- and sixteenth weeks of the study is shown in Table 6. There were no significant differences due to dietary treatments (Table 7) except during the eighth week, when the mean daily feed intake of CHOW (21.5 g) was significantly higher ($p < 0.05$) than the intakes of the experimental diets. Trends toward higher intakes of CHOW than of the experimental diets were observed during the fourth and sixteenth weeks, also. Based on observations during the three study periods, the incorporation of ammonium chloride, sodium chloride, or sodium and potassium bicarbonate did not affect feed intake of the rats in either age group. There were no significant differences in feed intake due to age (Table 8).

Since there was no interaction between age and diet on body weights and weight gains (Table 9), age groups were combined to study dietary effects and dietary treatements were combined to study age effects. As shown in Table 10, the highest weight at 36 weeks was observed in L-fed rats (711 g) and it was significantly higher ($p < 0.05$) than those for

TABLE 6

ANALYSIS OF VARIANCE OF MEAN DAILY FEED INTAKES

Source of Variation	df	Mean Squares		
		4th Week	8th Week	16th Week
Age	1	7	4	0
Diet	7	3.57	3.57[1]	3.71
"Error"	7	2.57	0.86	1.14

[1] $p < 0.05$

TABLE 7

EFFECTS OF DIETARY TREATMENTS ON MEAN DAILY FEED INTAKE[1] AT SELECTED WEEKS ON EXPERIMENT

Diet	Number		Feed Intake		
	Young	Old	4th Week g/d	8th Week g/d	16th Week g/d
CHOW	12	15	22.0±1.13[a] [2,3]	21.5±0.66[a]	21.0±0.75[a]
N	12	15	18.0±1.13[a]	17.5±0.66[b]	18.0±0.75[a]
NA	12	15	18.5±1.13[a]	18.0±0.66[b]	16.5±0.75[a]
NAB	12	15	19.0±1.13[a]	18.0±0.66[b]	17.5±0.75[a]
NBSALT	12	10	18.0±1.13[a]	17.5±0.66[b]	17.5±0.75[a]
L	12	15	18.5±1.13[a]	17.5±0.66[b]	17.5±0.75[a]
LA	12	15	19.0±1.13[a]	18.0±0.66[b]	17.5±0.75[a]
LAB	12	15	18.0±1.13[a]	18.0±0.66[b]	17.0±0.75[a]

[1]Mean intake per rat based on the mean consumption of rats in 3 cages, 4 or 5 rats per cage.

[2]Mean ± SE.

[3]Means in a column sharing a common superscript are not significantly different ($p < 0.05$) using Duncan's Multiple Range Test.

Table 8

EFFECT OF AGE ON MEAN DAILY FEED INTAKE[1] AT SELECTED WEEKS ON EXPERIMENT

Week	Feed Intake	
	Young (n = 96) g/d	Old (n = 115) g/d
4th	19.5 ± 0.57[2]	18.2 ± 0.57[3]
8th	18.7 ± 0.33	17.7 ± 0.33[3]
16th	17.7 ± 0.37	17.7 ± 0.37[3]

[1]Mean intake per rat based on the mean consumption of rats in 3 cages, 4 or 5 rats per cage.

[2]Mean ± SE.

[3]Not significantly different from young.

TABLE 9

ANALYSIS OF VARIANCE OF MEAN BODY WEIGHTS AND MEAN WEIGHT GAINS

Source of Variation	df	Mean Squares					
		0 Week Body Weight	4th Week Body Weight	8th Week Body Weight	16th Week Body Weight	36th Week Body Weight	Gain
Age	1	1248084.00[1]	889815.87[1]	716964.69[1]	614120.44[1]	485274.50[1]	176870.19[1]
Diet	7	1506.77	3495.10	3782.86	4298.59	11463.20[2]	16312.74[1]
Interaction	7	1133.43	2168.44	2327.75	2958.73	3103.01	1950.46
"Error"	132	1866.38	2068.13	2317.93	2755.34	4965.74	2956.88

[1] $P < 0.05$

[2] $P < 0.01$

TABLE 10

EFFECTS OF DIETARY TREATMENTS ON MEAN BODY WEIGHTS AND MEAN WEIGHT GAINS

Diet	Number		Body Weights					Weight Gain
	Young	Old	0 Week g	4th Week g	8th Week g	16th Week g	36th Week g	g
CHOW	9	9	518±10[a][1]	549±11[a]	568±11[a]	593±12[a]	643±17 [b][2]	125±13 [c]
N	9	8	496±10[a]	540±11[a]	566±12[a]	609±13[a]	691±17[ab]	195±13[ab]
NA	10	9	497±10[a]	517±10[a]	546±11[a]	588±12[a]	659±16 [b]	161±12 [bc]
NAB	10	9	504±10[a]	521±10[a]	542±11[a]	579±12[a]	655±16 [b]	151±12 [c]
NBSALT	10	6	492±11[a]	526±12[a]	552±12[a]	597±14[a]	692±18[ab]	200±14[a]
L	10	9	506±10[a]	554±10[a]	579±11[a]	622±12[a]	711±16[a]	205±12[a]
LA	10	10	516±10[a]	530±10[a]	549±11[a]	591±12[a]	658±16 [b]	142±12 [c]
LAB	10	10	502±10[a]	521±10[a]	539±11[a]	576±12[a]	647±16 [b]	145±12 [c]

[1]Mean ± SE.

[2]Means in a column sharing a common superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.

CHOW and acid-stressed rats (CHOW, 643 g; NA, 659 g and LA, 658 g), but it did not differ significantly from those for N- and NBSALT-fed rats (N, 691 g; NBSALT, 692 g). Body weights at 36 weeks of normal calcium and normal calcium, acid-stressed animals were not significantly different (N, 691 g vs. NA, 659 g), but low calcium and low calcium acid-stressed animals showed significant differences ($p < 0.05$) in body weights at 36 weeks (L, 711 g vs. LA,658 g).

The addition of sodium and potassium bicarbonate to the ammonium chloride-containing diets did not change body weights at 36 weeks, significantly. Weight gains showed a similar pattern of significant differences due to dietary treatments. Again, L-fed rats showed the highest weight gain (205 g), which was significantly higher ($p < 0.05$) than those for CHOW and acid-stressed rats (CHOW, 125 g; NA, 161 g; LA, 142 g), but it did not differ significantly from those for N- and NBSALT-fed rats (N, 195 g; NBSALT, 200 g). Also, in a like manner, weight gains of normal calcium and normal calcium, acid-stressed animals did not differ significantly (N, 195 g vs. NA, 161 g), but acid-stressed, low calcium-fed rats showed a significantly decreased ($p < 0.05$) weight gain (L, 205 g vs. LA, 142 g). Again, the addition of base to ammonium chloride diets did not change weight gain, significantly.

The effect of age on body weights and weight gains is presented in Table 11. Old rats were heavier than young rats throughout the study, but young rats showed significantly higher ($p < 0.01$) weight gain than old rats (200 g vs. 131 g).

Table 11

EFFECT OF AGE ON MEAN BODY WEIGHTS AT SELECTED WEEKS ON EXPERIMENT AND WEIGHT GAIN

Week	Body Weight	
	Young (n = 78) g	Old (n = 70) g
0	411 ± 5[1]	597 ± 5[2]
4th	454 ± 5	610 ± 5[2]
8th	485 ± 5	625 ± 6[2]
16th	529 ± 6	659 ± 6[2]
36th	612 ± 8	727 ± 8[2]
Gain	200 ± 6	131 ± 6[2]

[1]Mean ± SE.

[2]Significantly different from young ($p < 0.01$).

II. RENAL RESPONSES

When the urinary data were statistically analyzed, there was interaction between age and diet on all variables except volume (Table 12). Therefore the effects of diet within age groups, and the effects of age within each diet were compared. Table 13 shows the means and standard errors of five urinary parameters and comparison of diets within age groups and Table 14 presents the same means but shows the age comparisons within each diet.

As shown in Table 13, urinary pHs were significantly decreased ($p < 0.05$) in rats fed ammonium chloride-containing diets as compared with those of rats fed the same diets without ammonium chloride, regardless of age or level of dietary calcium (YN, 6.46 vs YNA, 5.57; ON, 6.23 vs. ONA, 5.69 and YL, 5.91 vs. YLA, 5.53; YL, 5.96 vs. OLA, 5.53). The addition of sodium and potassium bicarbonate to the ammonium chloride diets did not produce significant change in urinary pHs in either age group fed either level of calcium. Urinary pHs of young rats fed CHOW and NBSALT diets were significantly higher ($p < 0.05$) than those of young rats fed other control diets (YCHOW, 7.05 and YNBSALT, 6.89 vs. YN, 6.46 and YL, 5.91). In old rats urinary pHs were significantly different ($p < 0.05$) from each other on every control diet (OCHOW, 6.48; ON, 6.23; ONBSALT, 6.81: OL, 5.96). Except in the cases of CHOW- and N-fed rats (Table 14), urinary pHs were not significantly different between young and old rats (YCHOW, 7.05; OCHOW, 6.48; YN, 6.46; ON, 6.23).

TABLE 12

ANALYSIS OF VARIANCE OF URINARY VOLUME AND FIVE URINARY PARAMETERS

Source of Variation	df	Mean Squares					
		Volume	pH	Titratable Acidity	Ammonia	Calcium	Phosphorus
Age	1	145.16[1]	0.24[1]	96.12[1]	999.41[1]	2.24[1]	1643.04[1]
Diet	7	60.26[1]	5.20[1]	91.03[1]	1376.11[1]	5.96[1]	1089.41[1]
Interaction	7	21.62	0.24[1]	12.40[1]	134.06[1]	0.82[1]	145.24[1]
"Error"	132	11.40	0.03	1.38	15.55	0.28	37.01

[1] $P < 0.01$.

Table 13

EFFECTS OF DIET WITHIN AGE GROUPS ON FIVE URINARY PARAMETERS[1]

Group	n	pH	Titratable Acidity µEq/100 g body wt/hr	Ammonia µM/100 g body wt/hr	Calcium mg/24 hr	Phosphorus mg/24 hr
Young						
YCHOW	9	7.05[a2,3] ±0.06	0.81[f] ±0.39	2.31[c] ±1.31	0.86[b] ±0.18	12.72[e] ±2.03
YN	9	6.46[b] ±0.06	2.35[e] ±0.39	3.68[c] ±1.31	0.35[bc] ±0.18	20.10[d] ±2.03
YNA	10	5.57[d] ±0.06	5.98[cd] ±0.37	23.14[b] ±1.25	1.65[a] ±0.17	26.36[c] ±1.92
YNAB	10	5.64[d] ±0.06	6.80[bc] ±0.37	22.64[b] ±1.25	1.96[a] ±0.17	33.03[b] ±1.92
YNBSALT	10	6.89[a] ±0.06	1.14[f] ±0.37	3.15[c] ±1.25	0.46[bc] ±0.17	22.02[cd] ±1.92
YL	10	5.91[c] ±0.06	5.58[d] ±0.37	5.18[c] ±1.25	0.26[c] ±0.17	33.22[b] ±1.92
YLA	10	5.53[d] ±0.06	8.53[a] ±0.37	29.19[a] ±1.25	1.68[a] ±0.17	41.70[a] ±1.92
YLAB	10	5.55[d] ±0.06	7.57[ab] ±0.37	19.83[b] ±1.25	0.88[b] ±0.17	35.53[b] ±1.92

TABLE 13 (continued)

Group	n	pH	Titratable Acidity μEq/100 g body wt/hr	Ammonia μM/100 g body wt/hr	Calcium mg/24 hr	Phosphorus mg/24 hr
Old						
OCHOW	9	6.48[b] ±0.06	1.63[de] ±0.39	3.28[c] ±1.31	0.65[bc] ±0.18	13.42[c] ±2.03
ON	8	6.23[c] ±0.06	2.58[cd] ±0.42	2.94[c] ±1.39	0.35[c] ±0.19	21.29[b] ±2.15
ONA	9	5.69[e] ±0.06	3.39[bc] ±0.39	11.87[b] ±1.31	1.00[b] ±0.18	18.03[b] ±2.03
ONAB	9	5.61[e] ±0.06	3.61[bc] ±0.39	10.35[b] ±1.31	0.88[b] ±0.18	18.82[b] ±2.03
ONBSALT	6	6.81[a] ±0.07	1.00[e] ±0.48	3.05[c] ±1.61	0.40[c] ±0.22	17.34[c] ±2.48
OL	9	5.96[d] ±0.06	3.23[bc] ±0.39	3.72[c] ±1.31	0.20[c] ±0.18	23.23[b] ±2.03
OLA	10	5.53[e] ±0.06	6.20[a] ±0.37	20.87[a] ±1.25	1.89[a] ±0.17	35.65[a] ±1.92
OLAB	10	5.64[e] ±0.06	4.14[b] ±0.37	11.12[b] ±1.25	0.72[bc] ±0.17	22.15[b] ±1.92

[1]The value for each rat was based on 2, 72-hour urine collections.

[2]Mean ± SE.

[3]Means in a column sharing a common superscript are not significantly different (p 0.05) using Duncan's Multiple Range Test.

TABLE 14

EFFECT OF AGE WITHIN DIET GROUPS ON FIVE URINARY PARAMETERS[1]

Diet	pH		Titratable Acidity		Ammonia		Calcium		Phosphorus	
	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old
			µEq/100 g body weight/hour		µM/100 g body weight/hour		mg/24 hours		mg/24 hours	
CHOW	7.05	6.48[2,3]	0.81	1.63	2.31	3.28	0.86	0.65	12.72	13.42
N	6.46	6.23	2.35	2.58	3.68	2.94	0.35	0.35	20.10	21.29
NA	5.56	5.69	5.98	3.39	23.14	11.87	1.65	1.00	26.36	18.03
NAB	5.64	5.61	6.80	3.61	22.64	10.35	1.96	0.88	33.03	18.82
NBSALT	6.89	6.81	1.14	1.00	3.15	3.05	0.46	0.40	22.02	17.34
L	5.91	5.96	5.58	3.23	5.18	3.72	0.26	0.20	33.22	23.23
LA	5.53	5.53	8.53	6.20	29.19	20.87	1.68	1.89	41.70	35.65
LAB	5.55	5.64	7.57	4.14	19.83	11.12	0.88	0.72	34.53	22.15

[1]The value for each rat was based on 2, 72-hour urine collections.

[2]Mean.

[3]Means in a column sharing a common line are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.

As shown in Table 13, page 68, diets containing ammonium chloride resulted in significantly increased ($p < 0.05$) urinary titratable acidity (μEq/100 g body weight/hour) in young rats fed either level of calcium in the diet (YN, 2.35 vs. YNA, 5.98 and YL, 5.58 vs. YLA, 8.53). However titratable acidity of YNA rats (5.98) was not significantly lower than that of YL rats (5.58). In old rats, titratable acidity was increased in rats fed ammonium chloride-containing diets, but significantly so ($p < 0.05$) only when the diet was low in calcium (OL, 3.23 vs. OLA, 6.20). The addition of base to ammonium chloride diets containing normal levels of calcium (Table 13, page 68) did not significantly alter urinary titratable acidity in either age group of rats (YNA, 5.98 vs. YNAB, 6.80; ONA, 3.39 vs. ONAB, 3,61) while acid-stressed rats fed low calcium diets responded to the addition of base signigicantly ($p < 0.05$) (OLA, 6.20 vs. OLAB, 4.14). CHOW and NBSALT diets resulted in the lowest titratable acidities in both age groups (YCHOW, 0.81; YNSALT, 1.14; OCHOW, 1.63; YNSALT, 1.00), however in old rats titratable acidity of animals fed the CHOW-diet did not differ significantly from that of rats fed the N diet (2.58). With the exception of CHOW-, N-NBSALT-fed rats, titratable acidity was significantly lower in old animals than in young animals (Table 14, page 70).

Urinary ammonia (μM/100 g body weight/hour) increased significantly (Table 13, page 68) in acid-stressed rats in both age groups as compared with control rats both on normal (YN, 3.68 vs. YNA, 23.14; ON, 2.94 vs. ONA, 11.87) and low levels of calcium (YL, 5.18 vs. YLA, 29.19; OL, 3.72 vs. OLA, 20.87). The addition of base to low calcium

ammonium-chloride-containing diets significantly ($p < 0.05$) reduced ammonia excretion (YLA, 29.19 vs. YLAB, 19.83; OLA, 20.87 vs. OLAB, 11.12) but rats fed normal calcium diets did not exhibit significant decrements in urinary ammonia excretion upon base treatment. Urinary ammonia of rats of both age groups fed CHOW and NBSALT diets (YCHOW, 2.31; YNBSALT, 3.15; OCHOW, 3.28; ONBSALT, 3.05) did not differ significantly from that of rats fed other control diets containing either a low or normal level of calcium (YN, 3.68; YL, 5.18; ON, 2.94; OL, 3.72). Acid-stressed old rats excreted significantly less ammonia (Table 14, page 70) than similarly stressed young rats with or without the addition of base to the diets, regardless of calcium level.

Urinary calcium excretion (mg/24 hours) increased significantly ($p < 0.05$) (Table 13, page 68) in both young and old rats fed diets containing ammonium chloride, regardless of calcium level in the diet (YN, 0.35 vs. YNA, 1.65; ON, 0.35 vs. ONA, 1.00; YL, 0.26 vs YLA, 1.68; OL, 0.20 vs. OLA, 1.89). Incorporation of sodium and potassium bicarbonate into normal calcium, ammonium chloride-containing diets was without significant effect on urinary calcium excretion, however base incorporation into low calcium, ammonium chloride diets produced significant ($p < 0.05$) reductions in both age groups (YLA, 1.68 vs. YLAB, 0.88; OLA, 1.89 vs. OLAB, 0.72). In both age groups, urinary calcium excretion of animals fed CHOW diet was not significantly different from that of N- and NBSALT-fed rats. Calcium excretion in CHOW-fed old rats was not different from that of L-fed old rats, but CHOW-fed young rats excreted significantly ($p < 0.05$) more calcium than

L-fed young rats (YCHOW, 0.86 vs. YL, 0.26). Output of urinary calcium of NBSALT-fed rats was not significantly different from that of rats fed N and L diets, irrespective of age. As shown in Table 14, page 70, only rats fed NA and NAB diets demonstrated significant age differences ($p < 0.05$) in calcium excretion (YNA, 1.65 vs. ONA, 1.00; YNAB, 1.96 vs. ONAB, 0.88).

As shown in Table 13, page 68, urinary phosphorus excretion (mg/24 hours) was significantly ($p < 0.05$) increased in young animals fed ammonium chloride-containing diets regardless of calcium level (YN, 20.10 vs. YNA, 26.36; YL, 33.22 vs. YLA, 41.70). However, acid-stressed old rats showed significantly increased urinary phosphorus levels, only when the diet was low in calcium (ON, 21.29 vs. ONA, 18.03; OL, 23.23 vs. OLA, 35.65). Addition of base to diets containing normal levels of calcium of acid-stressed young rats significantly ($p < 0.05$) increased urinary phosphorus (YNA, 26.36 vs. YNAB, 33.03), but was without significant effect in old rats (ONA, 18.03 vs. ONAB, 18.82). Urinary phosphorus excretion was significantly decreased in both age groups by adding base to their low calcium diets (YLA, 41.70 vs. YLAB, 34.53; OLA, 35.65 vs OLAB, 22.15). CHOW-fed young rats excreted significantly less urinary phosphorus (12.72) than any other rats in that age group. Urinary phosphorus excretion of NBSALT-fed young rats (22.02) was not significantly different from that of rats fed the N diet (20.10), but it was significantly lower ($p < 0.05$) than that of rats fed the low calcium diet (33.22). In old rats, CHOW and NBSALT diets produced significantly lower ($p < 0.05$) levels of urinary phosphorus

excretion than any other dietary treatment; the difference between these two groups being non-significant (OCHOW, 13.42; ONBSALT, 17.34). Old rats, except those fed CHOW, N, and NBSALT diets excreted significantly less ($p < 0.05$) phosphorus in the urine than young rats (Table 14, page 70).

Since there was on interaction between age and diet on urinary volume (Table 12, page 67), age groups were combined to study dietary effects and dietary treatments were combined to study age effects. As shown in Table 15, urinary output (ml/24 hours) was lowest in rats fed the L diet (4.75), but it was not significantly different ($p > 0.05$) than that of N- and NBSALT-fed rats (N, 5.36; NBSALT, 6.92). Urinary output was highest in rats fed the LA diet (9.80) but it was not significantly different than that of rats fed the NA (7.55) or CHOW (8.05) diets. Sodium and potassium bicarbonate addition to normal calcium, ammonium chloride diets had no effect on urinary volume, but base addition to low calcium ammonium chloride diets significantly decreased urinary output (LA, 9.80 vs. LAB, 6.39). Urine volume was significantly higher ($p < 0.01$) in young rags (8.27) than in old rats (6.27).

Since statistical analysis showed no interaction between age and diet on kidney parameters (Table 16), age groups were combined to study dietary effects and dietary treatments were combined to study age effects. As shown in Table 17 there was a tendency for ammonium chloride to increase kidney weights (percent of body weight) in normal calcium rats (N, 0.47 vs. NA, 0.49) and the increase became significant ($p < 0.05$) in low calcium rats (L, 0.47 vs. LA, 0.55). Kidney weights

TABLE 15

EFFECTS OF DIETARY TREATMENTS ON URINE VOLUME

	Number		Urine Volume ml/24 hr
	Young	Old	
CHOW	9	9	8.05±0.80[abc][1,2]
N	9	8	5.36±0.82 [de]
NA	10	9	7.55±0.78[abcd]
NAB	10	9	9.35±0.78[ab]
NBSALT	10	6	6.92±0.87 [bcde]
L	10	9	4.75±0.78 [e]
LA	10	10	9.80±0.75[a]
LAB	10	10	6.39±0.75 [cde]

[1]Mean ± SE.

[2]Means sharing a common superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.

TABLE 16

ANALYSIS OF VARIANCE OF THREE KIDNEY PARAMETERS

Source of Variation	df	Mean Squares		
		Weight	PDG Activity	Protein
Age	1	0.0083	0.21	17.12[1]
Diet	7	0.0183[2]	96.21[2]	6.48
Interaction	7	0.0065	3.23	2.93
"Error"	132	0.0034	4.14[3]	3.89

[1] $P < 0.05$.

[2] $P < 0.01$.

[3] 131 df for error.

TABLE 17

EFFECTS OF DIETARY TREATMENTS ON KIDNEY PARAMETERS

Diet	Number		Kidney Weight % of Body wt	PDG Activity μM NH_3-N liberated/mg protein/hr	Kidney Protein mg/100 g tissue
	Young	Old			
CHOW	9	9	0.54±0.01^{a}[1,2]	8.26±0.48 ^{b}	16.32±0.46^{a}
N	9	8	0.47±0.01 ^{c}	7.47±0.49 ^{b}	16.86±0.48^{a}
NA	10	9	0.49±0.01 ^{bc}	12.49±0.47^{a}	15.83±0.45^{a}
NAB	10	9	0.49±0.01 ^{bc}	11.29±0.47^{a}	17.59±0.45^{a}
NBSALT	10	6	0.51±0.02^{abc}	7.18±0.52 ^{b}	17.12±0.51^{a}
L	10	9	0.47±0.01 ^{c}	8.43±0.47 ^{b}	16.68±0.45^{a}
LA	10	10	0.55±0.01^{a}	12.63±0.47^{a}	16.54±0.44^{a}
LAB	10	10	0.52±0.01^{ab}	11.80±0.45^{a}	15.93±0.44^{a}

[1]Mean ± SE.

[2]Means in a column sharing a common superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.

were high in CHOW-fed rats (0.54) and were intermediate in NBSALT-fed rats (0.51) with no significant differences existing between them and LA kidney weights (0.55). Inclusion of sodium and potassium bicarbonate in the ammonium chloride diets did not affect kidney weights, significantly at either level of calcium (NA, 0.49 vs. NAB, 0.49; LA, 0.55 vs. LAB, 0.52). Phosphate-dependent glutaminase activity (PDG) expressed as μM NH_3-N liberated/mg protein/hr) was increased significantly ($p < 0.05$) in rats fed ammonium-chloride diets (N, 7.47 vs. NA, 12.49; L, 8.43 vs. LA, 12.63). Incorporation of base into ammonium chloride diets resulted in non-significant reductions in PDG activities ($p > 0.05$). PDG activities were not significantly different from each other in control diet-fed animals (N, 7.47; L, 8.43; CHOW, 8.26; NBSALT, 7.18).

Kidney protein (mg/100 g tissue) was not affected by the dietary treatment (Table 17) but it was significantly higher ($p < 0.05$) in young rats (16.95) than in old rats (16.27). No significant differences due to age were demonstrated for kidney weight or PDG activity (Table 18).

Correlations among renal variables are shown in Figure 1. With the exception of the correlation of urinary pH with urinary volume, all renal variables were significantly correlated ($p < 0.01$). Kidney phosphate-dependent glutaminase activity showed significant positive correlation with urinary P, Ca, NH_3, TA and urinary volume, and showed significant negative correlation with urinary pH. Urinary pH was significantly positively correlated with kidney PDG activity. Urinary volume showed significant positive correlation with urinary P, Ca, NH_3, TA and kidney PDG. Urinary TA was significantly positively correlated

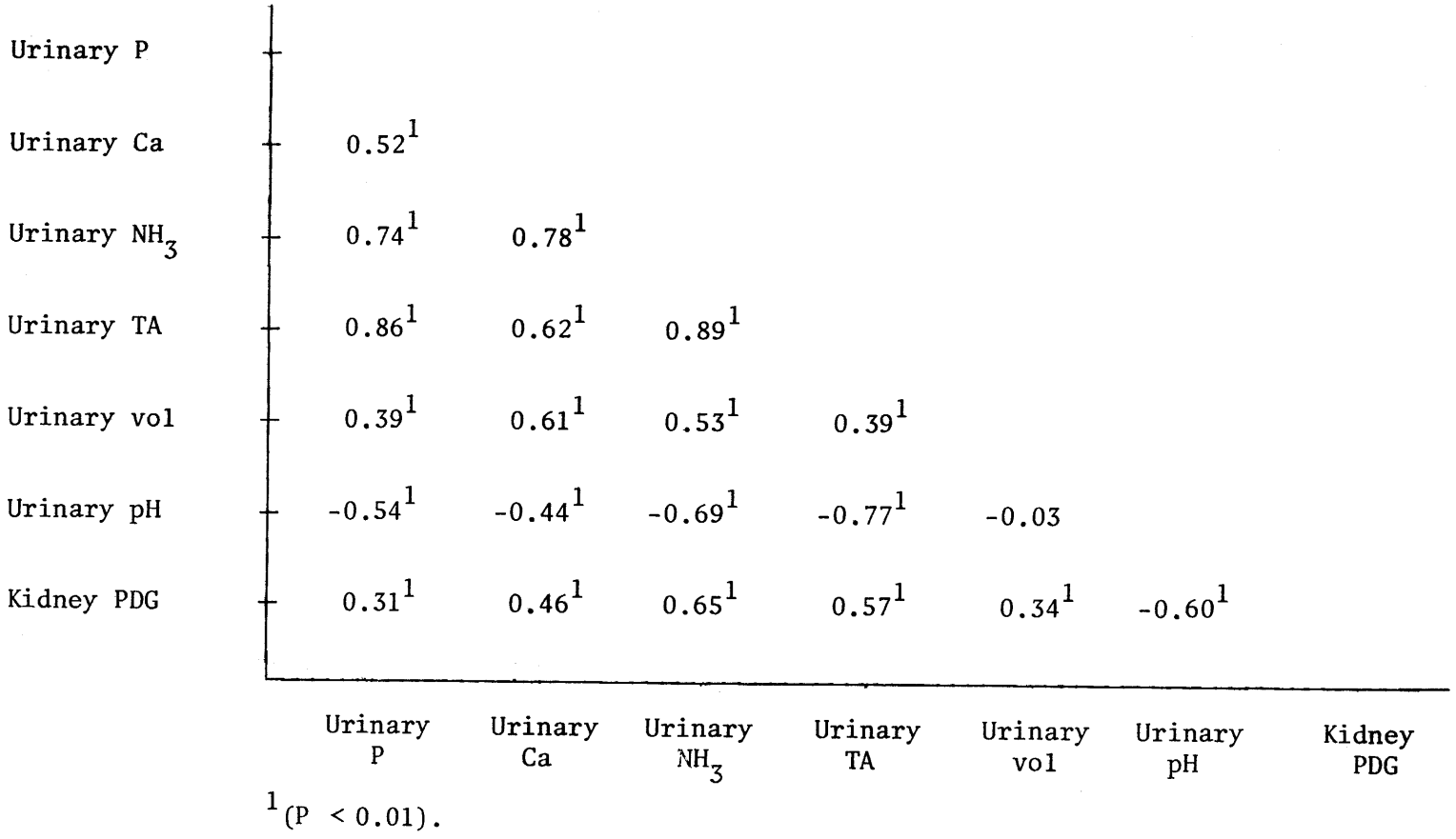
TABLE 18

EFFECT OF AGE ON KIDNEY PARAMETERS

Kidney Parameters	Age	
	Young (n = 78)	Old (n = 70)
Weight, % of body weight	0.511±0.007[1]	0.50 ±0.007
PDG Activity; μM NH_3-NH-N liberated/mg protein/hr	9.98 ±0.23	9.90 ± 0.25
Protein, mg/100 g tissue	16.95 ±0.22	16.27 ± 0.24[2]

[1]Mean ± SE.

[2]Significantly different from young ($p < 0.05$).



[1](P < 0.01).

Figure 1. Correlations Among Renal Parameters.

with urinary P, Ca, NH_3, volume and kidney PDG and significantly negatively correlated with urinary pH. Urinary ammonia showed significant positive correlation with urinary P, Ca, TA, volume and kidney PDG, and showed significant negative correlation with urinary pH. Urinary Ca was significantly positively correlated with urinary P, kidney PDG, volume, TA and NH_3 significantly negatively correlated with urinary pH. Urinary P showed significant positive correlation with kidney PDG, volume, TA, NH_3, and Ca, and showed significant negative correlation with urinary pH.

III. SERUM RESPONSES

Since there was no interaction between age and diet on serum calcium and phosphorus (Table 19), age groups were combined to study dietary effects and dietary treatments were combined to study age effects. Table 20 shows that acid stress had a tendency to decrease serum calcium (mg/100 ml) in rats fed normal calcium diets (N, 10.71 vs. NA, 10.54) and it significantly lowered serum calcium when the diet was low in calcium (L, 10.69 vs. LA, 10.28). There was a strong tendency for serum phosphorus (mg/100 ml) to be decreased in acid-stressed rats regardless of the level of calcium in the diet. The addition of base to diets containing ammonium chloride had no significant effects ($p > 0.05$) on either serum calcium or phosphorus levels in rats fed either level of calcium. The serum calcium level of CHOW-fed rats (10.30) was significantly lower ($p < 0.05$) than those of N- and L-fed rats (10.71, 10.69, respectively). Serum calcium and

TABLE 19

ANALYSIS OF VARIANCE OF SERUM CALCIUM AND PHOSPHORUS

Source of Variation	df	Mean Squares	
		Calcium	Phosphorus
Age	1	0.11	6.09[1]
Diet	7	0.54[2]	0.88[2]
Interaction	7	0.21	0.21
"Error"	131	0.20	0.37

[1] $p < 0.01$.

[2] $p < 0.05$.

TABLE 20

EFFECTS OF DIETARY TREATMENTS ON SERUM CALCIUM AND PHOSPHORUS

Diet	Number		Calcium mg/100 ml	Phosphorus mg/100 ml
	Young	Old		
CHOW	9	9	10.30±0.10 ^{b}[1,2]	5.91±0.14^{a}
N	9	8	10.71±0.11^{a}	5.69±0.15^{ab}
NA	10	9	10.54±0.10^{ab}	5.29±0.14 ^{b}
NAB	10	9	10.51±0.10^{ab}	5.40±0.14 ^{b}
NBSALT	10	6	10.46±0.12^{ab}	5.51±0.16^{ab}
L	10	9	10.69±0.10^{a}	5.57±0.14^{ab}
LA	10	10	10.28±0.10 ^{b}	5.29±0.14 ^{b}
LAB	10	10	10.30±0.10 ^{b}	5.33±0.14 ^{b}

[1]Mean ± SE.

[2]Means in a column sharing a common superscript are not significantly different ($p > 0.05$) using Duncan's Multiple Range Test.

phosphorus levels of NBSALT-fed rats (10.46, 5.51, respectively) did not differ significantly from those of rats fed any other diet. Serum phosphorus levels were highest in CHOW-fed rats (5.91), but they were not significantly different from those of animals fed N and L diets.

As shown in Table 21 age had no effect on serum calcium, but serum phosphorus was significantly lower ($p < 0.01$) in old rats (5.29) than in young rats (5.70).

IV. BONE RESPONSES

As shown in Table 22, tibia weights, showed significant differences in dry weights ($p < 0.05$) and dry, fat-free weights and fat content ($p < 0.01$) due to age, however, there were no significant, age-associated differences in ash, calcium or phosphorus contents. Also there were no significant differences in tibia analysis due to dietary treatments and no interaction between age and dietary treatment. Table 23 presents the effects of diet on tibia parameters, irrespective of age. Weights of tibias on a dry weight and fat-free basis ranged from a high of 0.7167 g and 0.6556 g, respectively in CHOW-fed to a low of 0.6624 g and 0.5954 g, respectively, in NA-fed rats. Tibia fat ranged from a low of 8.57% in CHOW-fed rats to a high of 10.62% in L-fed animals. Ash content of tibias ranged from a high of 70.82% in L-fed rats to a low of 70.10% in CHOW-fed animals. Tibia calcium content ranged from a high of 27.04% in NBSALT-fed rats to a low of 26.25% in NAB-fed animals. Phosphorus content of tibias ranged from a high of 12.56% in N- and NAB-fed rats to a low of 12.17% NBSALT fed rats. Neither fat, ash

TABLE 21

EFFECT OF AGE ON SERUM CALCIUM AND PHOSPHORUS

Analyses	Age	
	Young (n = 78)	Old (n = 70)
Calcium, mg/100 ml	10.50 ± 0.05[1]	10.45 ± 0.05
Phosphorus, mg/100 ml	5.70 ± 0.07	5.29 ± 0.07[2]

[1]Mean ± SE.

[2]Significantly different from young ($p < 0.01$).

TABLE 22

ANALYSIS OF VARIANCE OF TIBIA PARAMETERS

Source of Variation	df	Mean Squares					
		Dry wt	Fat-Free Dry Wt	Fat	Ash	Calcium	Phosphorus
Age	1	0.021[1]	0.035[2]	63.95[2]	2.21	1.92	0.05
Diet	7	0.006	0.008	6.89	1.54	1.35	0.39
Interaction	7	0.003	0.003	1.94	1.96	2.97	0.16
"Error"	132	0.005[3]	0.004[3]	4.38	2.44	4.26[3]	0.32[3]

[1] $p < 0.05$,

[2] $p < 0.01$,

[3] 131 df for error.

TABLE 23

EFFECTS OF DIETARY TREATMENTS ON TIBIA PARAMETERS[1]

Diet	Number Young	Number Old	Dry Weight g	Dry Fat-Free Weight g	Fat[2] %	Ash[3] %	Calcium[3] %	Phosphorus[3] %
CHOW	9	9	0.7167±0.0167[4]	0.6556±0.0152[4]	8.57±0.49[4]	70.10±0.37[4]	26.29±0.49[4]	12.34±0.13[4]
N	9	8	0.6797±0.0170	0.6111±0.0158	10.13±0.51	70.68±0.38	26.36±0.50	12.56±0.14
NA	10	9	0.6624±0.0161	0.5954±0.0148	9.98±0.48	70.81±0.36	26.36±0.47	12.46±0.13
NAB	10	9	0.6669±0.0161	0.6001±0.0148	9.97±0.48	70.59±0.36	26.25±0.47	12.56±0.13
NBSALT	10	6	0.6797±0.0182	0.6130±0.0167	9.82±0.54	70.34±0.40	27.04±0.53	12.17±0.15
L	10	9	0.6888±0.0161	0.6151±0.0148	10.62±0.48	70.82±0.36	26.66±0.47	12.21±0.13
LA	10	10	0.6985±0.0158	0.6307±0.0145	9.86±0.47	70.47±0.35	26.37±0.46	12.40±0.13
LAB	10	10	0.6657±0.0158	0.5966±0.0145	10.41±0.47	70.11±0.35	26.80±0.46	12.28±0.13

[1]Mean ± SE. [2]Based on dry weight. [3]Based on dry fat-free weight.

[4]The means in a column are not significantly different from each other ($p > 0.05$).

calcium nor phosphorus levels of tibias were affected by the level of calcium in the diet, acid stress or the addition of base.

Table 24 shows the effects of age on tibia analyses, regardless of diet. The dry weights of tibias of old rats were significantly less than those of young rats on a dry- (0.6703 vs. 0.6944, respectively) or dry fat-free basis (0.5992 vs. 0.6302, respectively). The tibias of old rats had significantly ($p < 0.01$) higher fat content (10.58) than tibias of young rats (9.26). The percentage of ash, calcium and phosphorus in the tibias of old rats tended to be higher than corresponding values in young rats.

TABLE 24

EFFECT OF AGE ON TIBIA PARAMETERS

Analyses	Age	
	Young (n = 78)[1]	Old (n = 70)
Dry wt, g	0.6944±0.0121[2]	0.6703±0.0121[3]
Dry fat-free wt, g	0.6302±0.0073	0.5992±0.0077[4]
Fat, %	9.26±0.24	10.58±0.25[4]
Ash, %	70.37±0.18	70.61±0.19
Calcium, %	26.40±0.24	26.63±0.25
Phosphorus, %	12.35±0.06	12.39±0.07

[1]77 young animals for dry weight, dry fat-free weight calcium and phosphorus.

[2]Mean ± SE.

[3]Significantly different from young ($p < 0.05$).

[4]Significantly different from young ($p < 0.01$).

CHAPTER V

DISCUSSION

I. RENAL ADAPTATION

The failure to obtain bone loss with acid stress can be explained, in part at least, by adaptative renal mechanisms mobilized in response to acid stress. These included decreased urinary pH, increased urinary titratable acid, ammonia, calcium, and phosphorus excretions, and increased kidney size and PDG activity. These adaptations were observed in rats in both age groups, however there were age-associated differences in most of the renal responses. For example, acid-stressed old rats excreted significantly less urinary titratable acid, ammonium and phosphorus than similarly treated younger rats. These observations resemble the findings of Adler et al. (81) who reported that total acid excretion, including ammonium, as well as phosphorus excretion was significantly reduced in elderly men as compared with younger men following ammonium chloride loading. In the present study, the contribution of urinary ammonium to acid excretion when expressed as a percent of total acid excretion (titratable acid plus ammonium) in young and old rats, 77% and 78% respectively, did not appear to differ significantly, although the differences were not analyzed statistically. This observation may indicate a species difference in urinary response to ammonium chloride loading, since Adler et al. (81)

observed that the percent urinary acid excreted as ammonium was significantly decreased in old subjects. They observed, also that total acid excretion, as well as ammonium excretion was reduced in elderly men during the control period. However in this study control values for titratable acidities and ammonium excretions did not differ significantly between young and old animals, suggesting that not until old rats were stressed did the difference in the handling of acid become apparent. The observation that old rats in this study did not differ significantly from younger animals in their ability to lower urinary pH under conditions of acid stress is similar to the results obtained by Adler et al. (81).

In contrast to the findings of Berg (83), urinary volume of young rats in the present study was significantly higher than that of old rats. Berg reported that urine output decreased up to 360 days of age and increased significantly thereafter. The explanation for the higher urinary outputs of young rats as compared to old rats in this study is not apparent, however the nature of the dietary treatments, and the fact that the daily urinary excretion of all components measured were either significantly higher or tended to be higher in young animals than in old ones, may have elevated the osmotic load to be excreted and obscured the expected increase in urinary output with advancing age. Lewis and Alving (74) found that the concentrating ability of the kidneys of human subjects, as indicated by specific gravity of the urine, declined with age.

The decreased excretions of titratable acid and ammonium in acid-stressed old rats in the present study suggests impairment of renal function with age. Since neither glomerular filtration rates nor DNA analyses of renal tissue were carried out, it is not possible to speculate as to the nature of the renal impairment, such as loss of nephrons or tubular defect. Gregory and Barrows (91) reported no age effect on glomerular filtration rates of rats in a study in which they employed clearance techniques similar to those used in human subjects to determine the effect of age on renal function. They interpreted their finding as indicating a minimal loss of nephrons with age, and stated that it compared well with the small age-dependent decrease in rat renal cells, based on DNA analyses reported by Barrows et al. (108). Further evidence for decreased tubular functions with age in rats has been reported by Adams and Barrows (89) and Beauchene et al. (90) who observed decreased active transport of para-aminohippurate (PAH) in kidney slices. The latter investigators also reported age-related decrements in the renal transport of α-amino isobutyrate (AIB) and of the activity of sodium-potassium stimulated ATPase. Spontaneous renal lesions have been reported in several strains of laboratory rats, including Sprague Dawley (109), Osborn-Mendel (110), and Wistar (109), and proteinuria has been observed in Sprague-Dawley rats by Gilson (109) and in Wistar rats by Beauchene et al. (85).

The observation that there was no age-associated difference in PDG activity in rats in the present study is again similar to the findings of Adler et al. (81). They reported that following the

ingestion of ammonium chloride and glutamine in young and old men, ammonium excretion levels increased in both groups with no significant difference in their increases. The observation that kidney protein concentration was lower in old than in young rats in this study is in agreement with the finding of Beauchene et al. (85) who reported decreased kidney protein concentrations with age in rats.

Since renal responses of rats to ammonium chloride loading in the present study resembled those which have been observed in human beings under similar conditions, it is conceivable that the acid-stressed animals were in positive hydrogen ion balance, however balance studies were not carried out which would substantiate this supposition. Lemann et al. (35) have called attention to the positive hydrogen ion balance and the persistent negative calcium balance in human subjects during ammonium chloride loading. They suggested that acidosis stimulates the exchange of hydrogen ions for other cations, calcium in particular in bone and that bone may provide a large buffer reserve which is available for removing hydrogen ions from the circulation during acidosis. Thus, bone buffering of hydrogen ions presumably accounts for the discrepancy between hydrogen ion production and urinary excretion in acidosis.

If it can be postulated that the acid-stressed rats in the present study were in positive hydrogen ion balance and that bone buffering could account for the discrepancy between acid production and excretion, then bone loss would have been expected. Furthermore if acid-stressed old rats were in more positive hydrogen ion balance than young rats,

as indicated by their decreased total acid excretion, then the bone loss should have been age-associated. However, bone composition of neither young or old acid-stressed animals differed significantly from that of rats fed control diets. This would imply that if the excess hydrogen ions were retained and buffered within the body, some tissue other than bone, or at least other than the tibia, supplied the base for neutralization, or that the acid was excreted via another route, such as feces. However, the latter route appears unlikely (34). The observation that tibia weights were significantly smaller in old rats, regardless of dietary treatments, indicating loss of bone with age, may suggest a non-nutritional cause of the bone loss, e.g. horomonal or possibly the absence of an unidentified nutritional factor in the diet necessary for bone maintenance in old animals. However, Draper and co-workers (11, 27, 28) have demonstrated that the development of osteoporotic changes in the skeleton of mice and rats, which are analogous to those observed in man, develop in spite of optimal allowances of all known essential nutrients including calcium, phosphorus fluoride and vitamin D.

II. DIET DIFFERENCES

In most respects the urinary responses of rats fed the low calcium diet resembled those of acid-stressed rats, although the changes were less pronounced. Compared to rats fed a diet containing a normal level of calcium, rats fed the low calcium diet had lower urinary pHs, higher excretions of titratable acid and phosphorus, and a

tendency toward increased ammonium excretion and PDG activities; however no renal hypertrophy was observed. The renal changes could have been due, in part at least, to the decreased alkalinity of the low calcium diet. In order to provide the normal and low levels of dietary calcium, calcium carbonate was included in the salt mixture of the normal calcium diet and omitted from that of the low calcium diet. Thus, with a less alkaline diet the animals adapted by lowering urinary pH and increasing titratable acid and ammonium excretions.

The addition of ammonium chloride to the low calcium diet intensified the renal responses observed on that diet. Or to make another comparison the renal responses of animals stressed with a low level of dietary calcium and ammonium chloride were greater than those of acid-stressed rats consuming normal levels of calcium.

The lowest pHs, the highest titratable acid, ammonium and phosphorus excretions and the highest PDG activities were observed in low calcium, acid-stressed rats. In addition, renal weights were highest and serum calcium and phosphorus levels were lowest in those animals. Despite the low level of dietary calcium and the acid stress, neither the tibia weight or its composition were significantly different from corresponding values of animals fed the normal calcium diet. It appears that acid-base homeostasis was maintained by renal mechanisms rather than by the withdrawal of mineral from the tibias to buffer the excess hydrogen ions produced by the ingestion of ammonium chloride.

The effects of incorporating sodium and potassium bicarbonate into the diets were most readily observable in the animals' renal responses.

The responses were exhibited in both age groups, but were most pronounced in old animals fed the low calcium diet as evidenced by significant decreases in titratable acid, ammonium, calcium and phosphorus excretions. Base addition also had a tendency to decrease PDG activities in acid-stressed animals. These findings are similar to those of Davies and Yudkin (51) who demonstrated that sodium bicarbonate decreased ammonium excretion in hydrochloric acid-stressed rats. Base addition had no significant effect on serum calcium and phosphorus levels nor did it change the ash, calcium or phosphorus levels of tibias.

The failure of prolonged ingestion of ammonium chloride by young and old rats in this study to produce significant bone loss is in disagreement with the results of Barzel and Jowsey (38) and Barzel (39). They reported that chronic ingestion of ammonium chloride by adult male rats resulted in significant bone loss in femurs, including both the organic and inorganic phases (38). They reported further that the changes were independent of calcium level of the diet. However, a reexamination of the data in their studies revealed that the losses in bone were due as much to the level of dietary calcium as to the acid stress. For example, when the level of calcium was 20 mg/100 g of diet bone losses occurred with or without an acid stress, but at 800 mg Ca/100 g of diet the bone losses were insignificant even with an acid stress.

In the present study, it appeared that 100 mg Ca/100 g of diet was sufficient to maintain calcium equilibrium in the rats in both age groups even in the presence of an acid stress. This observation is in

agreement with the finding of El-Maraghi et al. (111) who reported little if any effect on the weight of ash/cm^3 in the whole femurs of two-year old rats fed 12 weeks a diet that contained 110 mg Ca and 550 mg P/100 g. However, the results of the present study are in contrast to those of Larsson (12) who showed that ash content of whole tibias of adult male rats declined 6.2% after 16 weeks and 13.3% after 12 months when a diet containing 90 mg Ca and 550 mg P/100 g was fed. Larsson observed that 14 to 16 weeks were required for the development of a significant reduction in bone mass of adult rats, and he concluded that the study of El-Maraghi et al. (111) had not been of long enough duration. However, in the present study bone loss from tibias did not occur in rats fed the low calcium diet 9 months. Since it is known that the maintenance of normal bone in rats is dependent on many nutrients in the diet (93), differences in the results of the various studies could be due to other dietary factors. For example, Draper et al. (29) have reported osteoporosis in aging rats induced by high phosphorus diets. In addition, there could be strain differences in rats in susceptibility to bone loss, which could explain in part at least, the conflicting results. Perhaps it should be noted that bone loss did not occur in rats of Wistar origin fed a diet containing 100 mg Ca/100 g diet in this study, but it did occur in Sprague-Dawley rats fed a diet containing a similar level of calcium in the study of Larsson (12).

Numerous studies (16, 18, 19, 23) have demonstrated the ability of rats to adapt to a decreased intake of calcium, and the results of

this study substantiate that finding. Henry and Kon (16) showed that bone calcification in rats during the growth period was dependent on the calcium content of the diet, but at one year of age calcification was similar regardless of dietary level of calcium, and that it did not change during the next year. Henry et al. (18) concluded that the more efficient retention of calcium by rats ranging in age from 3 to 16 months fed a diet containing 0.13% Ca and 0.38% P was the result of decreased fecal excretion of calcium. Hironaka et al. (23) reported that endogenous excretion as well as intestinal absorption of calcium were significantly greater in aged rats (22 to 32 months) fed a diet containing 0.7% Ca and 0.45% P than in similarly fed younger rats (10 to 12 months). Although retention of calcium incorporated into the skeleton, as measured by Ca^{45} labeling, was significantly poorer in the aged animals than in younger ones, both groups were found to be in overall calcium balance. They suggested that aged rats were subject to greater loss of calcium from the body but were able to maintain calcium equilibrium by increasing the absorption of dietary calcium.

In the present study, the observation that serum calcium levels in rats fed the low calcium diet were not significantly different from those of rats fed the normal calcium diet, and that urinary calcium levels tended to decrease slightly as compared with those of rats fed the higher level of calcium, suggests that some parathyroid stimulation occurred in those animals. Harrison and Fraser (110) have reported increased parathyroid activity in rats maintained on a diet containing 8 mg Ca/100 g diet but otherwise adequate. Parathormone is known to

stimulate bone resorption, enhance calcium absorption from the intestine, increase urinary excretion of phosphate and enhance renal tubular reabsorption of calcium (111). If the 100 mg Ca diet fed to rats in this study led to parathyroid stimulation, it appeared to be mild, and it is possible that animals were able to maintain serum calcium levels by increasing tubular reabsorption of calcium without releasing calcium from bone. Peacock et al. (112) have suggested that the kidney is the most important target organ in the long-term regulation of serum calcium in human subjects.

Recently Draper et al. (29) reported significantly decreased weights of dry fat-free bone, ash, calcium and phosphorus of femurs, lumbar vertebrae and mandibles of 8 to 14 month-old rats fed diets which contained 0.6% Ca and either 1.2% P or 1.8% P. They attributed the bone resorption to excessive levels of dietary phosphorus which resulted in mild chronic secondary hyperparathyroidism due to a slight depression of plasma calcium associated with increased levels of circulating phosphate. Draper and his co-workers have proposed that the optimal Ca/P ratio for inhibition of bone resorption in adult animals is 2:1 (27). However, in the present study no bone loss was observed in either the young or old animals with a Ca/P ratio of 1.25:1 or 1:4. It is possible that the Ca/P ratio of 1:6 in the study of Larsson (12) and the 1:8 ratio in the diet fed to rats in the study of Barzel and Jowsey (38) contributed to bone loss. However, the effect of the level of calcium in the diet in the latter study cannot be dismissed.

Rats fed the NBSALT diet showed serum and renal responses which were quite similar to those of rats fed the normal diet. However, kidney weights of those animals receiving additional dietary sodium chloride were observed to be as high as those of low calcium, acid-stressed rats. Lotspeich (60) has reported similar findings.

There were some indications that the CHOW diet did not provide for as high a level of nutritional status in rats as did the normal calcium, semi-synthetic diet. For example, even though food intake tended to be highest in that group, weight gain was significantly lower than that of normal calcium diet-fed rats. However, the mean weight of their tibias tended to be higher while the mean percents ash and fat in their tibias tended to be lower than that of normal calcium diet-fed animals. CHOW-fed rats exhibited serum renal responses which closely resembled those of rats fed the normal calcium diet. However, their mean kidney weight was high and not significantly different from low calcium, acid-stressed rats. The latter observation may imply that solute load and protein content (24.5%) of the diet caused renal hypertrophy. Janicki et al. (59) have reported kidney enlargement in mice fed a high-protein diet.

Although it was thought that low levels of dietary calcium and prolonged ingestion of ammonium chloride by adult rats would lead to bone loss, no such bone losses occurred. The results indicated that renal adaptation to an acid stress and/or a low level of dietary calcium accounted for the failure to obtain bone loss. However, old rats demonstrated both age- and diet-associated decrements in urinary responses. In addition, old animals showed bone loss regardless of diet.

CHAPTER VI

SUMMARY

The effects of age and acid stress on bone and renal responses in 13 month- and 25-month old rats were investigated. In this study, neither organic nor inorganic phases of tibias were affected by dietary treatments in either age group of rats, but tibias of old animals weighed significantly less and their fat content was significantly higher than that of younger animals.

Serum phosphorus levels were significantly higher in young rats. Acid-stressed animals showed significant decreases in urinary pH and significant increases in urinary titratable acid, ammonium, calcium, and phosphorus excretions, with age-associated decrements in old rats except urinary pH. Renal hypertrophy was observed in acid-stressed animals, as well as significantly increased phosphate-dependent glutaminase activities; however these changes were not age-associated. The concentration of kidney protein was significantly lower in old rats than in younger animals, regardless of dietary treatment. The effects of incorporating sodium and potassium bicarbonate into the diets containing ammonium chloride were most pronounced in old animals fed the low calcium diet as evidenced by significant decrements in the urinary responses.

It was concluded that rats in both age groups adapted to the metabolic acidosis by renal mechanisms, but that there was renal

impairment in old rats as evidenced by their decreased ability to excrete the acid load. Although the urinary responses of acid-stressed animals were affected by the calcium level of the diet, the animals in both age groups adapted to the low level of dietary calcium and bone composition of their tibias was not significantly altered.

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VITA

Grace Kathleen Newell was born in Stafford, Kansas, where she received her elementary and secondary education. She attended Kansas State University, Manhattan, Kansas and received the Bachelor of Science degree in Home Economics with a major in Dietetics and Institution Management in 1944. The following year she completed a dietetic internship at the University of Michigan Hospital, Ann Arbor, Michigan, and became a member of The American Dietetic Association. In 1951 she obtained a Master of Science degree in Home Economics with a major in Nutrition from the University of Wisconsin, Madison, Wisconsin. Before starting graduate study at The University of Tennessee she held positions as a hospital dietitian for ten years in Michigan and Colorado, four years as Assistant Professor in the Department of Home Economics at the University of Wyoming, Laramie, Wyoming, where she became a member of the American Home Economics Association, and eight years as Assistant Professor in the Department of Foods and Nutrition at Kansas State University. In the academic year of 1969-1970 she was granted sabbatical leave from her position at Kansas State University to work towards the Ph.D. degree at The University of Tennessee. During the following years of 1970 to 1972 she was granted additional leave to continue work towards the degree. She received the Ph.D. degree with a major in Nutrition from The University of Tennessee in August, 1973.