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The Effect of Dietary Protein and Calorie Restriction on Growth, Kidney Function, and Survival of Male Rats

Teresa Ann Davis
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Teresa Ann Davis entitled "The Effect of Dietary Protein and Calorie Restriction on Growth, Kidney Function, and Survival of Male Rats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Roy E. Beauchene, Major Professor

We have read this dissertation and recommend its acceptance:

Jane R. Savage, Frances E. Andrews, James M. Liles

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Frances C. Andrews

James M. Liles

Accepted for the Council:

L. Evan Pond

Vice Chancellor
Graduate Studies and Research

THE EFFECT OF DIETARY PROTEIN AND CALORIE RESTRICTION ON GROWTH, KIDNEY FUNCTION, AND SURVIVAL OF MALE RATS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Teresa Ann Davis

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ABSTRACT

Growth, kidney function, and survival were studied in male Wistar rats fed for 1 (12 animals per dietary group) or 2 (36 animals per dietary group) years low, medium, and high protein diets at 2 caloric levels and diets fed ad libitum but gradually altered in protein content during growth and development. Restricted groups (R) fed 18, 30, and 42% casein diets were provided two-thirds of the mean quantity of diet consumed by groups fed 12, 20, and 28% casein ad libitum (A), respectively, and thus consumed the same amount of protein but one-third less calories. Two other groups were fed ad libitum initially a 20% casein diet which was either decreased to a 12% casein level or increased in casein content to 28% by 1 year of age.

Caloric restriction significantly decreased the mature body weight and rates of weight gain at 10 and 20 weeks of age but increased growth rate (k) as calculated from the Brody growth equation using body weights obtained at 16 points during the first year of life. While dietary protein level had no significant effect on mature body weight, it significantly affected the rate at which mature body weight was attained. High dietary protein levels were generally associated with high k values at both caloric levels, however, R animals fed a medium protein diet had the highest k value. Dietary protein level had a greater effect on rates of weight gain in A than in R groups and was positively associated with rate of weight gain during the early growth period (10 weeks of age) but during the later growing period (20 weeks of age) was negatively

associated. Animals fed varying levels of protein ad libitum had higher mature body weights and rates of weight gain than those fed a constant level of protein and generally consumed more diet. Animals fed a constant low level of protein tended to consume a smaller quantity of diet than those fed higher dietary protein levels.

Measurements made in this study for tests of renal function were urinary protein excretion and volume, in vitro transport of para-aminohippuric acid (PAH) by kidney slices, and kidney DNA content. Age and diet comparisons were made on 1 (young) and 2 (old) year animals. Age significantly increased urinary volume and protein excretion, decreased PAH transport, and had no effect on renal DNA content. Caloric restriction delayed the age-associated decline in renal function as indicated by a decrease in urinary protein excretion and an increase in PAH transport in R as compared to A groups. Young and old animals fed restricted diets excreted larger volumes of urine than those fed ad libitum. Caloric intake had no effect on renal DNA content. Urinary protein excretion and volume significantly increased with dietary protein level in old animals. There was also a significant quadratic effect of protein and a quadratic protein-calorie interaction for urinary protein excretion in old animals, i.e., old animals fed ad libitum a medium protein diet had the highest mean protein excretion value. Dietary protein level had no significant effect on PAH transport. While there was no significant effect of protein level in the diet on kidney DNA content, there was a significant linear interaction of protein and calories in old animals; DNA values were positively associated with dietary protein level in old ad libitum fed groups but negatively

associated in those fed restricted diets. There was a significant negative correlation between proteinuria and renal PAH transport in most old ad libitum-fed groups indicating either variable could be used as an indication of renal function. While caloric restriction had the greatest effect on slowing the renal aging process, protein restriction also was beneficial in delaying the age-associated decline in renal function.

Caloric restriction significantly increased the percent survival of animals at 1.5 and 2 years of age. While dietary protein level was positively associated with survival at 2 years, the survival rate of rats fed high protein diets at both caloric levels was less than or similar to that for R and A animals fed medium protein diets.

In general, caloric restriction decreased mature body weight and produced physiologically younger animals as indicated by an improvement in renal function and an increase in survival at 2 years. The effect of protein restriction was not of the same magnitude as caloric restriction and although it reduced the growth rate and favorably influenced renal function, it adversely affected survival. While high levels of dietary protein were detrimental to the function of a specific biological system, i.e., the kidney, medium or moderately high protein diets must be beneficial for other systems in these animals and therefore favorably influence survival.

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CHAPTER I

INTRODUCTION

One of the primary goals of nutrition research is to determine the levels of various nutrients which are needed to promote rapid mental and physical growth, optimal physiological performance during maturity, and the retention of good health during old age (1). It is generally considered that the intake of nutrients at levels moderately above those required for maximum rates of growth and development are optimum for the well-being of an organism. However, it has been shown that life span can be increased and the age-associated decline in physiological function delayed in experimental animals fed diets containing lower levels of nutrients than those required for optimal growth and development.

There is much controversy as to the relative effect of decreased caloric and protein intake on life span of experimental animals (2). It is generally accepted that the reduction of feed intake increases longevity. However, the results of studies which investigated the effect of dietary protein on life span are equivocal and the level of caloric intake was not comparable between dietary protein levels. In addition, the reduction of caloric intake in most studies was accompanied by a decrease in protein intake.

Restriction of calories and/or protein has been reported to decrease the incidence of renal lesions in old rats and delay the age-associated decline in renal function (3). While a reduction in urinary protein excretion and an improvement in the transport of

para-amonohippuric acid has been demonstrated in calorically restricted animals, the effect of dietary protein intake on these biochemical parameters has not yet been determined.

It was the purpose of this study to investigate the effects of protein restriction with and without caloric restriction on growth and survival of rats and to determine the effects of these diets on renal function. In addition, the effect of gradually changing the level of dietary protein during growth and development without reducing caloric intake on these parameters was studied.

It was hypothesized that caloric restriction would reduce rates of growth and mature body weights and produce physiologically younger animals as indicated by improved renal function and increased survival. It was further hypothesized that protein restriction with or without caloric restriction would reduce rates of growth but not mature body weights and produce physiologically younger animals.

CHAPTER II

REVIEW OF LITERATURE

I. EFFECTS OF FEED RESTRICTION ON GROWTH, HEALTH, AND LONGEVITY

A. TOTAL DIETARY RESTRICTION

The effect of feed restriction on the life span of animals was studied as early as 1935 by McCay and coworkers (4) who severely retarded the growth of rats by feed restriction throughout life. They found that feed restriction significantly increased the life span of the experimental animals. In a subsequent experiment, rats were retarded in growth by extreme underfeeding until 300, 500, 700, or 1000 days of age (5). In general, the length of life was directly proportional to the length of time for which growth was retarded.

In a series of investigations, Berg (6) and Berg and Simms (7,8) studied the effect of feed restriction on growth, disease incidence, and longevity. Rats were restricted from weaning to either 54 or 67% of ad libitum feed intake. While the shapes of the growth curves for restricted animals were similar to those fed ad libitum, the mature body weights attained were less and their overall health and fertility were better (6). The incidences of cardiac, renal, and vascular lesions and tumors throughout adult life and old age of restricted animals were significantly less than that of unrestricted animals (7,8). The life spans of restricted animals were extended about 25% beyond those fed ad libitum.

Since severe dietary restriction during early life may lead to a marked reduction in growth and biochemical and physiological maturation, several investigators have studied the effect of time of introduction of feed restriction on life span. In general, these studies (9-11) have shown that the effect of dietary restriction on longevity depends upon the severity and length of restriction and the stage of the life cycle in which it is imposed.

Ross (9) reported that rats restricted to 6 grams of diet per day from weaning had the greatest life span. However, when the same level of restriction was imposed at 10 months or at 1 year, the length of life was substantially less than controls fed ad libitum. A less severe restriction of 8 or 10 grams imposed at 10 months or 1 year increased life span.

Nolen (10) fed rats diets restricted to 60 or 80% of ad libitum feed intake throughout life, after 12 weeks of ad libitum feeding, or until 12 weeks of age with ad libitum feeding thereafter. He reported that restricted rats consuming 60% of ad libitum feed intake throughout life or before 12 weeks of age had life spans significantly greater than rats fed the other diets. However, he recommended the 80% of ad libitum intake regime from weaning as it resulted in increased longevity without "altering the physiological and chemical profiles of the animals."

The effects of dietary restriction imposed during the first year of life, thereafter, or throughout life have been studied by 2 research teams (11,12). Although their results were not identical, in general, they concluded that animals restricted during any stage of life lived longer than those fed ad libitum throughout life.

Stuchlikova et al. (11) found a greater life span in rats, mice, and golden hamsters fed restricted diets during the first year of life and then fed ad libitum than in those fed restricted diets throughout life. The life span of animals fed ad libitum for 1 year and thereafter fed restricted diets was greater than that of animals restricted throughout life but less than the life span of those fed restricted and then fed ad libitum. Animals fed ad libitum throughout the life span had the lowest survival rate. Although animals restricted for 1 year then fed ad libitum had the greatest longevity, they were also the most obese.

Tucker et al. (12) restricted rats by feeding them 15 out of each 48 hour period throughout life or during or after the first year of life. The mean life span of animals restricted throughout life was significantly greater than those of all other dietary groups.[1] Animals restricted during either the first year of life or thereafter had similar life spans but were significantly greater than for those fed ad libitum throughout life. A comparison of restricted with ad libitum-fed animals showed a lower growth rate and mature body weight for restricted animals (about 70% of ad libitum) (12). At 2 years of age restricted-fed rats had significantly less body fat than ad libitum-fed animals.[1] The body fat content of rats restricted during only the second year of life was similar to those restricted the first 2 years of life and that for animals restricted the first year of life and then fed ad libitum was similar to those fed ad

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[1]Beauchene, R. E., Bales, C. W., Smith, C. A., Tucker, S. M. & Mason, R. L. (1979) The effect of feed restriction on body composition and longevity of rats. Physiologist 22, 8.

libitum for 2 years. Old animals restricted-fed during the second year of life had similar body compositions to 1 year old ad libitum or restricted-fed rats. In addition, all restricted groups had improved kidney function (12).

B. DIETARY PROTEIN LEVEL

Restriction of dietary protein has also been shown to increase longevity and delay age-associated physiological changes and disease processes. Leto et al. (13,14) reported that the life span of mice fed 4% protein diets ad libitum was extended beyond those fed 26% protein diets ad libitum. Miller and Payne (15) found that rats fed a diet containing 12% protein for 120 days and a 4% protein diet thereafter exhibited a greater life span than those fed either 12, 8, or 4% protein diets throughout life. The longest-lived group had slower growth rates and were less obese than the 12 or 8% protein fed groups.

Goodrick (16) examined the relationships between growth characteristics and longevity in various strains of mice fed diets containing either 4 or 26% casein. In 2 or 3 strains the life spans of animals fed the low protein diet were increased. A slower growth rate and increased growth duration were associated with an increased life span. A high body weight was not related to increased mortality; longevity was positively correlated to peak body weight but negatively correlated to body weight 1 month prior to death. He suggested that it is the slowed growth rate and the resultant longer growth duration which are responsible for the increased longevity in restricted animals rather than the reduction of body weight.

While the previous studies reported an increased life span in animals fed protein restricted diets, other investigators have found little or no effect of dietary protein level on longevity. Barrows and Kokkonen (17) reported that lowering the dietary protein level from 23 to 12% at 16 months of age increased the longevity of rats, whereas decreasing it to 8 or 4% was not effective. Nakagawa et al. (18) examined the effect of dietary protein level on growth, longevity, and incidence of lesions in rats. During the early growing period, growth rate was positively associated with dietary protein level but by 6 months the differences in body weights were insignificant. There was no effect of diet on longevity or on the number of lesions observed at death.

There is much controversy as to the relative effect of protein versus caloric restriction on longevity. Barrows (19) has suggested that it is the reduction in protein intake rather than that of calories which results in the increased longevity. He hypothesized that dietary restriction increases longevity by decreasing the use of the genetic code thereby reducing genetic imperfections in senescence.

A few studies have compared the aging effects of various dietary protein levels under ad libitum and restricted feeding conditions. Ross and Bras (20) showed a direct relationship between dietary protein level and longevity in groups of rats fed either ad libitum or calorically restricted diets. Restricted animals had greater life spans than ad libitum-fed animals and the differences in life spans between dietary protein groups were more pronounced in restricted-fed animals. Fernandes et al. (21) showed caloric restrictions in a short-lived mouse strain susceptible to immunocomplex

nephritis. However, protein restriction was more effective than caloric restriction in another short-lived strain. A subsequent study by the same authors showed that mice calorically restricted from weaning or at 4 or 5 months of age or those fed protein restricted diets at weaning were protected against the development of immune nephritis (22).

It is difficult to compare the effects of calorie and protein restriction on longevity since the caloric intake of rats fed various dietary protein levels ad libitum may vary (14). Additionally, in most studies in which caloric restriction was utilized to increase longevity, the reduction in caloric intake was accompanied by a decrease in protein intake (4-12). However, the dietary regimes used by Visscher et al. (23) were either a control diet fed ad libitum or one containing an equal amount of protein but one-third less calories. He reported a higher percent survival and a lower incidence of spontaneous mammary carcinoma in mice fed the restricted diet.

The effect of dietary protein to carbohydrate ratio on longevity has been investigated by Ross and Bras (24) in rats allowed to select the amount of dietary protein, carbohydrate, and calories consumed throughout life. They reported that rats which consumed relatively low amounts of protein and high quantities of carbohydrate in early life and high protein, low carbohydrate diets in late life had the shortest life spans. The longest-lived rats consumed a moderately high protein to carbohydrate ratio throughout life. Regardless of the composition of the diet, caloric intake was inversely correlated with longevity.

II. EFFECT OF DIET ON AGING IN THE KIDNEY

A. RENAL AGE CHANGES

It is widely accepted that the structure and function of the kidney are altered during aging (3,25-37). Since these age changes are similar in humans and rats, the most remarkable exception being the absence of atherosclerosis in rat renal blood vessels, the rat has been used extensively as an experimental model to study aging in the kidney and it is primarily a summary of these studies which will be presented here.

While renal mass decreases with age in humans following maturation (25,26), age changes in rat kidney weight vary (3). In addition, there is a decrease in the number of nephrons in the old kidney and these appear to be morphologically different from young nephrons (3,26). Glomerulonephritis and glomerulosclerosis commonly occur in old rats, with a thickening of the glomerular and tubular basement membranes and a proliferation of the epithelial and masengial cells (3,26,27). Further age changes include a thickening and fusion of the epithelial cell foot processes, the deposition of eosinophilic material in the tubule lumina, and an increase in interstitial connective tissue (3,26-29).

Numerous studies indicate that renal function decreases with age. Glomerular filtration rate as measured by inulin or creatinine clearance decreases with age (25,26) as well as urine concentrating ability (30). The ability of old rat kidneys to accumulate para-aminohippuric acid (PAH) and alpha-aminoisobutyric acid declines with age indicating a possible decrease in the membrane transport of organic anions with age (31).

Perhaps one of the most pronounced age-associated changes in renal function of rats is a marked increase in urinary protein (32,33). Beauchene et al. (34) also reported an increased urinary protein excretion and an increased albumin synthesis by the liver with age in rats. They postulated that the increased synthesis of albumin was a direct result of the loss of albumin in the urine. While other researchers have also shown an increased serum albumin synthesis with age (35-37), Obenrader et al. (37) suggested that the increased albumin synthesis is not a consequence of increased albumin excretion but rather that the opposite may be true. Their evidence included an increase in urinary protein excretion without a stimulation of albumin synthesis following the administration of aminonucleoside puromycin, a drug which causes renal damage.

While renal function declines during aging, the activities of most renal enzymes generally remain unchanged. Burich (30) studied the effect of age on renal enzyme activities of mice and found that in old as compared to mature animals, there was a decrease in lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities but no changes in the activities of Na-K-ATPase and glutaminase with age. Barrows and Roeder (38) reported little change in the activities of succinoxidase, pyrophosphatase, and alkaline phosphates but an increase in cathepsin activity in kidneys of 24 month old rats as compared to 12 month old animals. Leto et al. (13) also found an increase in cathepsin activity with age but no change in renal LDH, MDH, and succinoxidase with age. Wilson and Franks (39) studied the effect of age on renal enzyme activities of pre-weanling and 6, 18, and 30 month old mice. They found little age change in the

activities of acid phosphatase, B-glucuronidase, LDH, succinic dehydrogenase, and cytochrome oxidase, an increase in glucose-6-phosphate dehydrogenase activity, and a decrease in the activities of glucose-6-phosphatase and 5'-nucleotidase at 18 months. The activity of kidney catalase was reported by Stoltzner (40) to decrease with age.

Kidney DNA content and protein synthesis have been reported to be relatively unaltered by age. Both Barrows and Roeder (38) and Leto et al. (13) found only a slight decline in kidney DNA content from maturity to old age. Beauchene et al. (34) reported no significant differences in the incorporation of ^{14}C from 2-^{14}C-acetate and ^{3}H from UL-^{3}H-isoleucine into kidney protein of old as compared to mature rats.

B. DIET AND RENAL FUNCTION

Restriction of calories or protein has been reported to reduce the prevalence of renal disease and delay age-associated changes in renal function. In a study by Berg and Simms (7), the incidence of glomerulonephritis in rats restricted to 54% of ad libitum feed intake was 6% at 1200 days of age as compared to 67% in ad libitum fed animals. Bras and Ross (41) reported a direct relationship between the level of protein in the diet and the prevalence of glomerulonephrosis in male rats fed either 10, 22, or 51% casein diets.

Both Bras (42) and Saxton and Kimball (43) compared the effects of caloric and protein restriction on renal lesions. Bras (42) fed rats diets containing various levels of protein and calories

from weaning and concluded that both protein and caloric restriction decreased the incidence of glomerulonephrosis. Saxton and Kimball (43) also reported that either type of dietary restriction had a beneficial effect in rats but suggested that calories appeared to have the greater effect. Other researchers fed rats diets ranging in protein content from 10 to 36% and found no effect of diet on renal lesions (18).

In a study on the effect of intermittent feeding on growth and renal function of male rats, Tucker et al. (12) reported improved renal function in feed restricted animals. Urinary protein losses of rats fed ad libitum throughout life were greater than that for animals restricted during either the first, second, or both years of life. Old animals restricted during either the first or the second year of life had similar urinary protein losses but greater than that of animals restricted for 2 years. At 2 years of age, ad libitum-fed animals also had a higher incidence and severity of renal lesions than restricted animals and these were positively correlated to urinary protein excretion. PAH transport in 2-year old rats was significantly less in ad libitum-fed animals than in animals restricted-fed during any part of life.

Kleinknecht et al. (44) studied the effects of 14, 27, and 37% protein diets on growth, renal function, and survival of rats partially nephrectomized at 1 month of age. In uremic rats, high protein diets were associated with a decreased weight gain and increased serum creatinine level at 3 months of age and an increase in mortality rate. However, in control rats at 3 months of age, dietary protein had little effect on renal function and was positively associated with growth.

Few studies have been conducted on the effect of diet on renal enzyme activities and morphology. Stoltzner (40) fed mice diets containing 4, 8, or 24% protein from weaning or young adulthood and found catalase activity to be unaffected by diet. Dietary protein restriction has been reported by Leto et al. (13) and Barrows and Kokkonen (45) to significantly decrease the activities of LDH, MDH, and succinoxidase in the kidney. Intermittently-fed mice that were fasted prior to sacrifice had decreased activities of these 3 enzymes whereas intermittently-fed animals fed prior to sacrifice showed increased enzyme activities (45). Mean enzyme values of the intermittent-fed and intermittent-fasted animals were similar to mice fed normal levels of dietary protein. The activities of these enzymes were unaffected by age but the activity of renal cathepsin was increased (13).

In the same laboratory, the morphology of the mouse kidney glomeruli was studied by electron microscopy in animals fed normal protein diets ad libitum or intermittently and in mice fed 4% protein diets ad libitum.[2] Glomerulas diameter decreased with age but was reduced by caloric or protein restriction.

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[2]Johnson, J. E. & Barrows, C. H. (1979) Scanning electron microscopic observation on aging and dietary restricted mouse kidney glomeruli. Gerontologist 19, 96.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Male Wistar rats were obtained as weanlings from the National Research Laboratories in Creve Coeur, Missouri. At 32 days of age, animals were assigned to dietary groups so that the mean weights of each group were similar. Of the animals to be killed at 2 years of age (old), 36 animals were assigned to each dietary group. When the old animals were 1 year of age, 12 weanling animals were assigned to the same diets and were killed at 1 year of age (young).

Animal groups are shown in Table 1. Restricted groups (R) fed 18, 30, and 42% casein diets were provided two-thirds of the mean quantity of diet consumed by groups fed 12, 20, and 28% casein diets ad libitum (A), respectively. Two other groups were fed ad libitum (A) initially a 20% casein diet which was either decreased or increased in casein content by 0.8% per month for 10 months. The latter groups were maintained thereafter at a constant dietary casein level of 12 or 28%, respectively, until sacrificed. The composition of the 20% casein diet is shown in Table 2. Diets were made isocaloric by substitution of equal amounts of corn starch and sugar for casein.

All rats were individually housed in 7" x 10" x 7" wire mesh stainless steel cages. The positions of cages within the rack and racks within the room were rotated weekly. Water was provided ad libitum. Restricted groups were provided two-thirds of the mean amount of feed consumed by their ad libitum-fed controls. Daily feed intake was monitored on approximately one-third of the animals

TABLE 1

DIETARY TREATMENTS

Dietary protein designation	Casein in diet	
	Ad libitum-fed	Restricted-fed[1]
	%	%
Low	12	18
Medium	20	30
High	28	42
Medium/low[2]	20 → 12	
Medium/high[2]	20 → 28	

[1]Restricted animals were fed two-thirds of the mean feed intake of their ad libitum-fed controls.

[2]Percent dietary casein was changed 0.8% per month for 10 months and maintained at constant levels thereafter.

TABLE 2

COMPOSITION OF 20% CASEIN DIET

Dietary component	Percent of diet
Casein[1,2]	20.0
Sucrose	29.0
Cornstarch	29.0
Crisco	6.0
Wesson oil	2.0
Vitamin mix[1,3]	2.0
Salt mix[1,4]	3.0
Alphacel[1]	9.0

[1]Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

[2]Determined by the Kjeldahl method to contain 91.5% protein.

[3]Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin mix): vitamin A, 4.5; vitamin D, 0.25; thiamin hydrochloride, 1.0; riboflavin, 1.0; niacin, 4.5; p-aminobenzoic acid, 5.0; calcium pantothenate, 3.0; pyridoxine hydrochloride, 1.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.00135; alpha-tocopherol, 5.0; and sufficient dextrose to make 1 kg.

[4]Formulated to supply the following amounts of minerals (g/kg salt mixture): $CaCO_3$, 543.0; $MgCO_3$, 25.0; $MgSO_4$, 16.0; NaCl, 69.0; KCl, 112.0; KH_2PO_4, 212.0; $FePO_4 \cdot 4H_2O$, 20.5; KI, 0.08; $MnSO_4$, 0.35; NaF, 1.00; $Al_2(SO_4)_2K_2SO_4$, 0.17; and $CuSO_4$, 0.90.

in each ad libitum-fed group on a rotating basis until animals were 1 year of age. Thereafter, restricted feeding was based on the mean biweekly feed consumption of the ad libitum-fed controls.

Rats were weighed weekly until 6 months of age, every other week from 6 to 12 months of age, and monthly thereafter. Rats were killed at either 1 or 2 years of age and were designated young and old, respectively.

II. URINE COLLECTION

Animals were placed in metabolism cages and urine collected for 72 hours at 12 and 24 months of age. Bottles containing 0.2 ml thymol in propanol (10% w/v) were placed in 600 ml beakers and positioned under the metabolism cage funnels so that the urine flowed into bottles and the feces into beakers (46). Each day the funnels were rinsed with approximately 0.3 ml water delivered from a wash bottle and the collection bottles replaced. The urine was centrifuged (Model V, International Equipment Co., Boston, Mass.) daily for 10 minutes at 800 x g to separate the diet from the urine. Urine volume was measured and corrected for the funnel rinsings. At the end of the 72 hour collection period, the urine was filtered and frozen until analyzed.

III. URINARY PROTEIN DETERMINATION

The sample size to be used for the quantitative analysis of urinary protein was determined by estimating the degree of turbidity formed by adding sulfosalicylic acid to urine (47). Biuret reagent was used for the color development in which complex ions are formed

in an alkaline solution between proteins and copper to give a purple color (48,49).

Reagents.

1. Sulfosalicylic acid, 3% (w/v): 3 g $HO_3S \cdot C_6H_3(OH)CO_2H$ was dissolved and diluted to 100 ml with water.

2. Sodium chloride, 5% (w/v): 5 g NaCl was dissolved and diluted to 100 ml with water.

3. Perchloric acid (PCA), 0.14% (w/v): 0.4 ml 70% $HClO_4$ was diluted to 200 ml with acetone immediately before use.

4. Trichloroacetic acid (TCA), 5% (w/v): 25 g $Cl_3C \cdot CO_2H$ was dissolved and diluted to 500 ml with water.

5. Sodium hydroxide, 0.1 N: 2 g NaOH was dissolved and diluted to 500 ml with water.

6. Biuret reagent: 3 g $CuSO_4 \cdot 5H_2O$, 9 g $NaKC_4H_4O_6$ $4H_2O$, 8 g NaOH, and 5 g KI were added and dissolved one at a time and diluted to 1 liter with water.

7. Bovine serum albumin standard: 1 g bovine serum albumin (BSA) was dissolved and diluted to 100 ml with 5% NaCl. Working standards were prepared daily by diluting 0.0 - 0.8 ml stock standard to 1 ml with 5% NaCl.

Procedure. To determine the urine sample size, 5 drops 3% sulfosalicylic acid was added to 5 drops of urine. If a slight turbidity developed, 0.8 ml urine was used; if a light precipitate was observed, 0.6 ml urine was used; and if a heavy precipitate developed, 0.4 ml urine was used. All samples and standards were diluted to 1.0 ml with 5% NaCl.

Four ml of 0.14% PCA in acetone was added to samples and standards and mixed. The contents of the tubes were incubated for 20 minutes in a 50^{o} water bath and centrifuged (Model V, International Equipment Co., Boston, Mass.) at 800 x g for 12 minutes. The supernatant was discarded, 2 ml 5% TCA was added to the pellet, and the contents of the tubes were mixed, centrifuged for 10 minutes at 800 x g, and the supernatant removed. The protein pellets were dissolved in 1 ml 0.1 N NaOH by incubation in a 37^{o} water bath for 15 minutes. Four ml of biuret reagent was added and the contents of the tubes were mixed and further incubated at 37^{o} for 15 minutes. The absorbances were measured in a spectrophotometer (Model 24, Beckman Instruments, Palo Alto, Ca.) at 550 nm. Protein excretion per 24 hours was calculated as follows:

$$\frac{\text{mg protein}}{\text{24 hr}} = \frac{\text{Conc std}}{\text{A std}} \times \text{A sample} \times \frac{1}{\text{sample vol}} \times \text{24 hr urine vol}$$

IV. PREPARATION OF TISSUE

Each rat was stunned by a blow to the head and decapitated using a guillotine. Kidneys were removed, placed on chilled watch glasses and decapsulated. The left kidney was weighed to the nearest one-hundreth g and frozen until analyzed for DNA content. The right kidney was weighed to the nearest 0.2 mg and kept on ice until sliced for PAH determination.

V. PARA-AMINOHIPPURIC ACID TRANSPORT

The ability of the kidney to perform osmotic work was measured by the active transport of para-aminohippuric acid (PAH) from a

suspending medium into kidney slices. The PAH accumulated into kidney slices was determined by diazotization and a subsequent reaction which yielded a purple color that was measured spectrophotometrically (50).

Reagents.

1. Medium, pH 7.3: 5.06 g NaCl, 2.98 g KCl, 1.36 g sodium acetate ($C_2H_3NaO_2 \cdot 3H_2O$), 1.98 g sodium phosphate (Na_2HPO_4 $7H_2O$), and 0.2000 g PAH ($NH_2C_6H_4CONHCH_2COOH$) were dissolved in 800 ml water, and the pH was adjusted to 7.3 with 2 N HCl. The solution was then diluted to 900 ml with water. A 0.11% (w/v) calcium chloride ($CaCl_2 \cdot 2H_2O$) solution was mixed with medium immediately before use at a ratio of 1:9.

2. Sodium nitrite, 0.2% (w/v): 0.2 g $NaNO_2$ was dissolved and diluted to 100 ml with water immediately before use.

3. Ammonium sulphamate, 0.5% (w/v): 0.5 g $NH_4O_3SNH_2$ was diluted to 100 ml with water.

4. N-(1-naphthyl)-ethylene diamine dihydrochloride (EDA), 0.2% (w/v): 0.2 g $C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$ was dissolved and diluted to 100 ml with water.

5. Hydrochloric acid, 0.1 N: 4.3 ml HCl was diluted to 500 ml with water.

6. Physiological saline, 0.9% (w/v): 9 g NaCl was diluted to 1 liter with water.

7. Trichloroacetic (TCA), 10% (w/v): 50 g $Cl_3C \cdot CO_2H$ was diluted to 500 ml with water.

8. Hydrochloric acid, 2 N: 17.2 ml HCl was diluted to 100 ml with water.

9. Para-aminohippuric acid (PAH) standard: 0.1000 g $NH_2C_6H_4CONHCH_2COOH$ was dissolved in 100 ml 0.1 N HCl. Ten ml of this standard was diluted to 100 ml with 0.1 N HCl. The final working standard was prepared by diluting 10 ml of the second standard to 100 ml with 0.1 N HCl to obtain a final concentration of 10 μg/ml. A series of standards was prepared fresh daily by diluting 0.0 - 2.5 ml of the working standard to 6 ml with 0.1 N HCl.

Procedure. Ten ml of the medium including the calcium chloride was pipetted into each of a series of 25 ml Erlenmeyer flasks which had been chilled on ice in the shaker bath rack. The right kidney of each animal was sliced with a Stadie-Riggs microtome equipped with a 0.5 mm head (Arthur H. Thomas, Philadelphia, Pa.). The outer slices from each side were discarded while the 2 inner slices from each side were weighed to the nearest 0.2 mg and immediately dropped into a chilled flask containing the medium. Slices were incubated for 1 hour at 37^{o} in a shaker water bath (Eberbach Corp., Ann Arbor, Mich.) oscillating at a rate of 120 cycles per minute. Oxygen was bubbled into the medium of each flask throughout the incubation.

After incubation, the rack was removed from the water bath and placed on ice. Slices were removed from the flasks with forceps, dipped in physiological saline, and drained against the sides of the container. Each slice was placed in a graduated centrifuge tube until homogenized. Slices were homogenized in about 1 ml water using a tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa.) with a Teflon pestle. The homogenizing vessel and pestle were rinsed with 10% TCA after each use and the final volume of the

homogenate was brought to 7 ml in the graduated centrifuge tubes with 10% TCA. Samples were mixed and centrifuged (Model V, International Equipment Co., Boston, Mass.) at 800 x g for 10 minutes.

Duplicate 2 ml aliquots of the homogenate supernatants were pipetted into graduated centrifuge tubes and 0.5 ml of 2 N HCl was added. After adding 2 ml water, tubes were capped with aluminum foil and placed in a boiling water bath for 30 minutes. Samples were cooled in an ice bath following hydrolysis.

The following reagents were added in order to each sample and standard: 0.5 ml sodium nitrite, 0.5 ml ammonium sulphamate, and 0.5 ml EDA. The contents of the tubes were mixed after the addition of each reagent and the final volume of the samples was brought to 7.5 ml with 0.1 N HCl.

Samples and standards were allowed to stand for 10 minutes and the absorbances were measured in a spectrophotometer (Model 24, Beckman Instruments, Palo Alto, Ca.) at 540 nm. PAH transport was determined using the following equation:

$$\mu g \text{ PAH/g tissue} = \left(\frac{\frac{\text{Conc std}}{\text{A std}} \times \text{A sample} \times 3.5}{\text{g tissue}} \right) - 200$$

PAH values were also expressed as μg PAH/mg DNA.

VI. DNA DETERMINATION

Tissue DNA was isolated by a modification of the Schmidt-Tannhauser method (51). Tissue homogenates were extracted with cold acid and the residues digested in alkali to solubilize the RNA. The tissue DNA and protein were then precipitated by the addition of acid. The amount of DNA in the extract was estimated by the Ceriotti method

(52) in which DNA is reacted with indole and the color measured spectrophotometrically.

Reagents.

1. Perchloric acid (PCA), 1.2, 0.6, and 0.2 N: to prepare the 1.2 N solution, 51.5 ml of 70% $HClO_4$ was diluted to 500 ml with water. 0.6 N and 0.2 N solutions were prepared by diluting 25.7 and 8.6 ml, respectively, of 70% PCA to 500 ml with water.

2. Potassium hydroxide, 0.3 and 0.1 N: to prepare 0.3 and 0.1 N solutions, 8.42 and 2.81 g, respectively, of KOH were dissolved and diluted to 500 ml with water.

3. Indole reagent: 40 mg C_8H_7N was added to 25 ml water and placed in a boiling water bath until dissolved. Sixty µl 0.1 M $CuSO_4$ was added and the final volume brought to 100 ml with water.

4. DNA standard: 40 mg DNA was dissolved in 100 ml water (400 µg/ml) and refrigerated. A 100 µg/ml working standard was prepared fresh daily by diluting 1.5 ml of the stock standard to 6 ml with 0.1 N KOH. A series of standards was prepared by diluting 0.0, 0.2, 0.4, 0.8, and 1.0 ml of the working standard to 2 ml with 0.1 N KOH.

Procedure. Five ml of a 5% (w/v) kidney homogenate (Polytron, Brinkman Instruments, Westbury, N.Y.) prepared in cold water was pipetted into duplicate centrifuge tubes. To each tube was added 2.5 ml cold 0.6 N PCA. The contents of the tubes were mixed and allowed to stand on ice for 10 minutes. The samples were centrifuged (HN-S, Damon/IEC Division, Needham Hts., Mass.) for 10 minutes at 1000 x g and the supernatants discarded. The samples were kept in ice throughout the DNA isolation procedure except where otherwise

indicated. The precipitates were washed twice by mixing with 5.0 ml cold 0.2 N PCA and then centrifuged for 10 minutes at 1000 x g. The supernatants were discarded and after the last wash, the tubes were inverted over filter paper.

To each tube was added 4.0 ml of 0.3 N KOH and the pellet broken with a stirring rod. The tubes were capped and incubated for 1 hour at 37^{o}. After cooling in an ice bath, 2.5 ml of 1.2 N PCA was added and the tubes were allowed to stand in the ice bath for 10 minutes. The precipitates were washed twice with 0.2 N PCA as previously described.

To each tube was added 5.0 ml of 0.3 N KOH and the precipitate broken with a stirring rod. The tubes were capped and incubated at 50^{o} for about 1 hour, i.e., until the precipitate was dissolved. The samples were poured into 50 ml volumetric flasks, 12 ml 0.3 N KOH was added, and the contents of the flasks brought to volume with water. Duplicate 2.0 ml aliquots of each hydrolyzed tissue sample were pipetted into centrifuge tubes. To each standard and sample was added, in order, 1.0 ml indole reagent and 1.0 ml concentrated HCl, and the contents of the tubes were mixed after each addition. The tubes were capped and placed in a boiling water bath for 10 minutes. After cooling on ice, 4.0 ml chloroform was added and the contents of the tubes carefully mixed for 15 seconds. The standards and samples were centrifuged for 5 minutes at 500 x g and the pink bottom layers aspirated from the yellow upper layers. Each yellow layer was again extracted with 4.0 ml chloroform and the formers absorbances then determined in a spectrophotometer

(Model 24, Beckman Instruments, Palo Alto, Ca.) at 490 nm. Renal DNA content was calculated using the following equation:

$$\text{mg DNA/g tissue} = \left(\frac{\overline{X}\ \text{Conc std}}{\overline{X}\ \text{A std}} \times \text{A sample} \right) \div 10$$

VII. SURVIVAL

Cages were checked daily for animal deaths. All animals were autopsied after death and any gross pathological conditions noted. Percent survival was calculated for each dietary group at 1, 1.5, and 2 years of age.

VIII. BRODY GROWTH CURVES

Using the equation developed by Brody (53), growth curves were calculated by computer from 16 body weights obtained during each animal's first year of life. Growth curves were calculated as follows:

$$W = A - Be^{-kt}$$

where W = Animal weight (g) at any given age;

A = mature weight (g);

B = integration constant;

e = base of natural logarithms;

k = rate of growth with respect to the growth yet to be made; and

t = age (weeks).

A, B, and k values were calculated for each animal and used to determine mean growth cruves for each group. Rates of growth at 10 and 20 weeks were calculated from individual k and B values using the equation rate = kB^{tk}.

IX. STATISTICAL METHODS

The experimental plan of this study was to estimate the effects of 1) calories (A vs R), 2) protein (linear and/or quadratic for protein), and 3) the interaction of protein and calories (linear and/or quadratic interaction) on growth, renal function, and percent survival. Procedures available in GLM (general linear models) of SAS 1979 were used to complete these analysis (54). Analysis of variance was used to test differences among dietary treatments and age (55). Specific differences among group means were tested with statistical contrasts (56). Probability levels (α-risks) of less than 0.10 were considered statistically significant. All A and R groups were included in the test of effects of calories if no differences were found between A animals fed a constant level of protein and those fed diets in which percent protein in the diet was altered (constant protein A vs variable protein A). When differences were found between the two types of A feeding, only groups fed a constant level of protein were compared to R groups to determine caloric effects. Group means and standard errors were estimated with least square means options available in GLM. Correlation coefficients were calculated for renal function measurements. The influence of diet on percent survival was tested using FUNCAT (functional analysis of catagorical variables), an option available in SAS.

CHAPTER IV

RESULTS

Figure 1 shows the calculated growth curves for all restricted and ad libitum-fed animals (R and A, respectively) provided constant levels of dietary protein throughout life. Growth curves were drawn using the mean values calculated for mature body weight, integration constant, and relative growth rate, i.e., A, B, and k, respectively, in the Brody equation (53), for each dietary group. The shapes of the calculated growth curves for A groups were similar to those for R groups but at a lower level. Initially, the growth curve of rats fed throughout life a low protein diet ad libitum was lower than those for other A groups but by 1 year the curves were similar. Figure 2 shows calculated growth curves for ad libitum animals fed diets decreased or increased in dietary protein level during the first year of life. Rats fed ad libitum initially a medium protein diet decreased to a low dietary protein level by 1 year of age had the highest growth curve.

Means of growth curve variables for each dietary group are shown in Table 3. Also presented in Table 3 are the results of utilizing statistical contrasts to determine the effect of constant (12, 20, and 28%) versus variable (20 → 12 and 20 → 28%) levels of dietary protein in animals fed ad libitum, caloric restriction, dietary protein level in A and R groups (linear and/or quadratic effect), and the interaction of calories and protein in A and R groups (linear and/or quadratic interaction) on growth curve variables. Since significant differences were found between growth curve

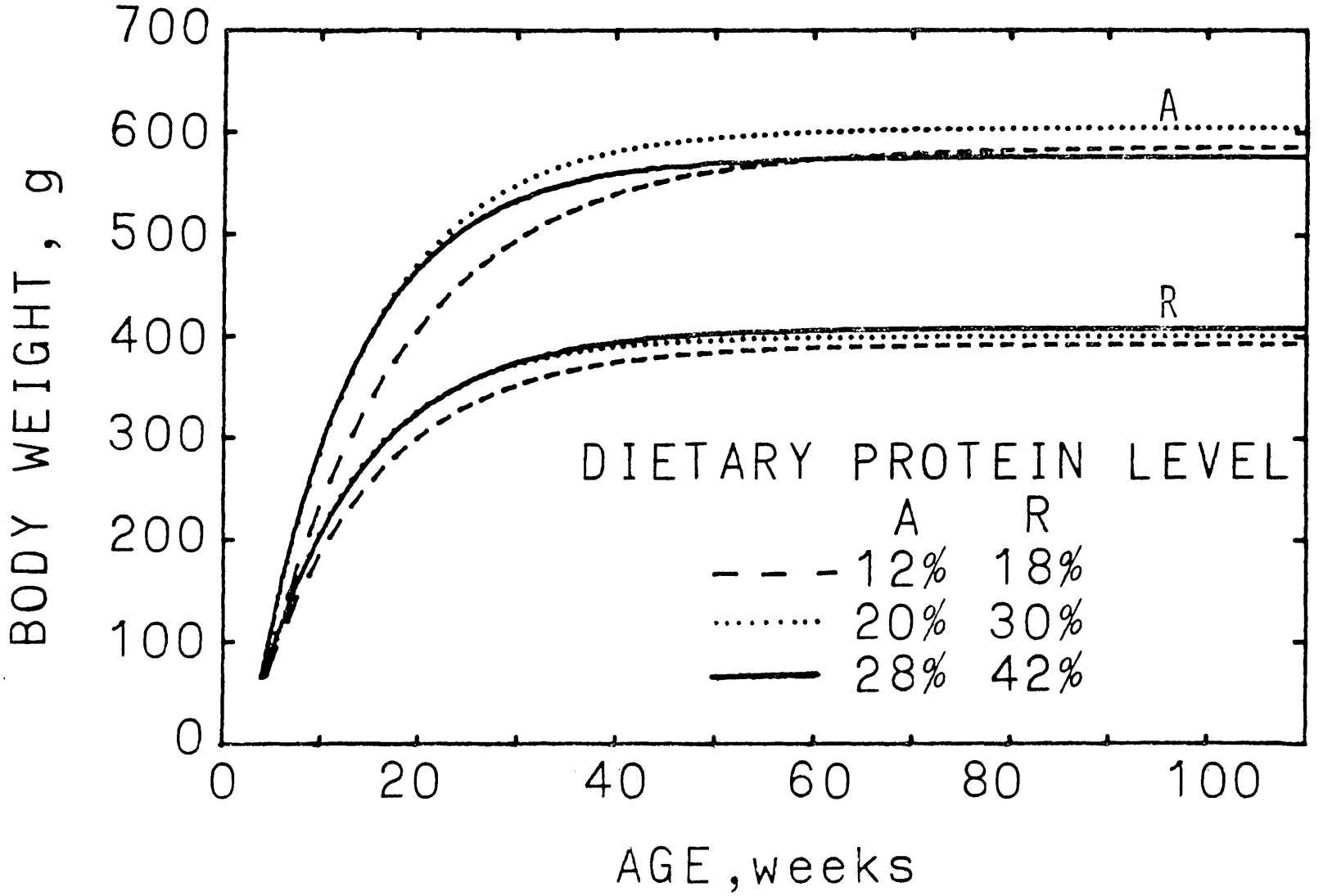

Figure 1. Influence of feeding low, medium, and high levels of dietary protein on an ad libitum (A) or restricted (R) basis on calculated growth curves.

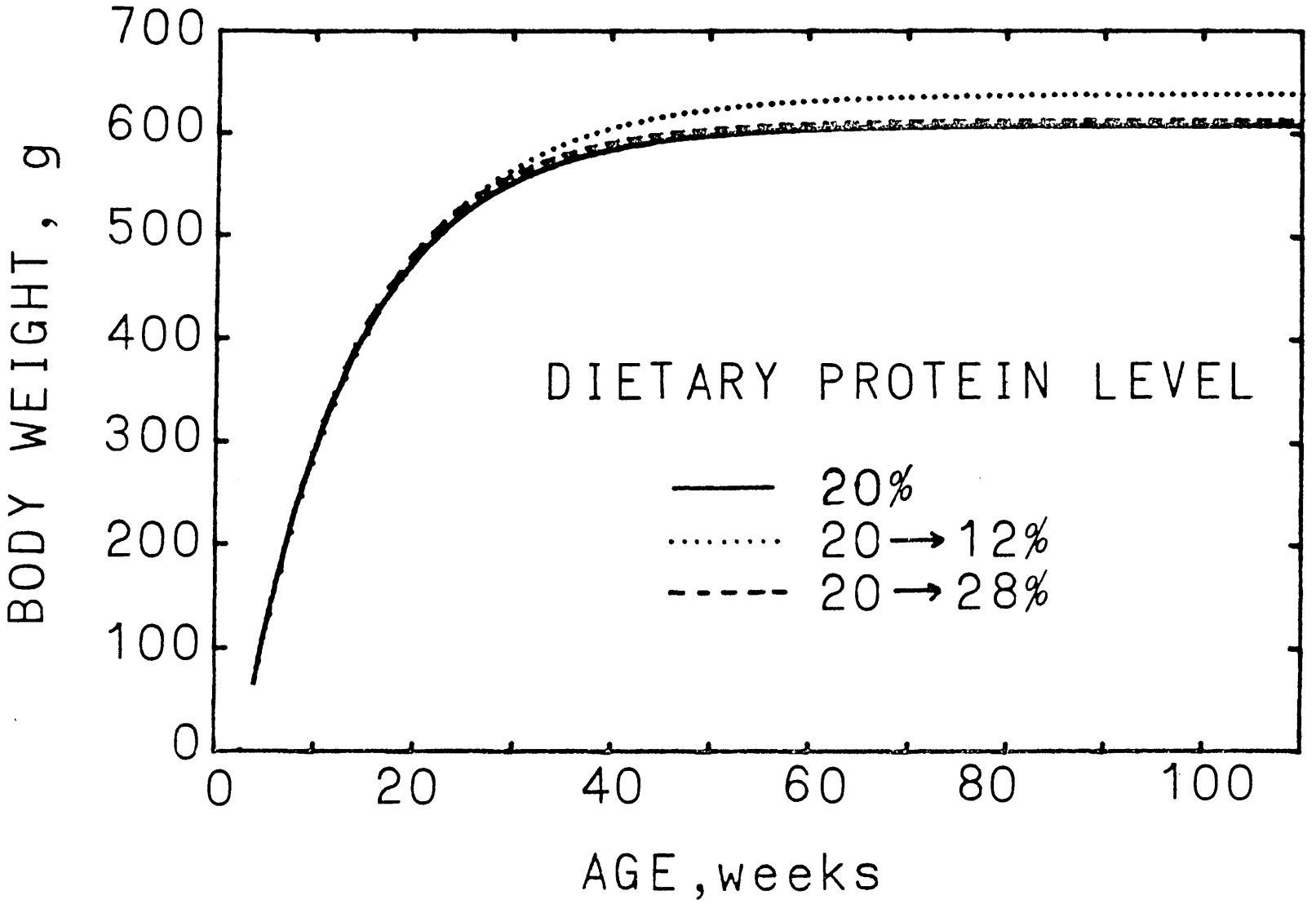

Figure 2. Influence of feeding constant and varying levels of dietary protein ad libitum on calculated growth curves.

TABLE 3

MEANS AND SIGNIFICANCE LEVELS FOR CONTRASTS OF GROWTH CURVE VARIABLES BY DIET

Treatment	Predicted mature wt[1]	Growth rate[2]	Wt gain 10 wk	Wt gain 20 wk
	g		g/wk	g/wk
Percent protein				
Ad libitum				
12	574.7	0.0680	23.0	11.7
20	607.7	0.0861	26.6	11.5
28	569.0	0.0946	26.4	10.4
20 → 12	638.9	0.0779	27.1	12.6
20 → 28	613.3	0.0885	27.9	11.7
Restricted				
18	388.8	0.0810	16.4	7.3
30	397.0	0.0966	18.4	7.0
42	403.8	0.0889	17.8	7.3
SEM for column	±13.3	±0.0025	±0.4	±0.3
Contrast statements		Significance[3]		
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	***	NS	***	***
Ad libitum vs restricted	***	***	***	***
Linear for protein	NS	***	***	*
Quadratic for protein	NS	***	***	NS
Linear interaction	NS	***	**	*
Quadratic interaction	NS	NS	NS	NS

[1]Predicted mature body weight, A in Brody equation (53).

[2]Growth rate, k in Brody equation (53).

[3]Statistical significance for contrast statements are indicated by *($p < 0.10$), **($p < 0.05$), and ***($p < 0.01$). Contrast statements not statistically significant are indicated by NS ($p > 0.10$).

variables of animals fed constant levels of protein and those fed variable levels of protein ad libitum, variable protein groups were not included in the A vs R contrast.

Mean predicted mature body weights (A in Brody equation) ranged from 569.0 to 638.9 g in animals fed ad libitum and were significantly greater ($p < 0.01$) than those of R groups which ranged from 388.8 to 403.8 g. Dietary protein level had no effect on predicted mature weights, but did affect the growth rate (k). Growth rates in groups fed ad libitum ranged from 0.0680 to 0.0946 with animals fed a low protein diet at a constant level throughout life and those fed a diet decreased from a medium to a low protein level having the lower values. R groups had k values which ranged from 0.0810 to 0.0966. The overall effect of protein on growth rate was both linear and quadratic but the interaction of protein and calories was linear ($p < 0.01$). High dietary protein levels were generally associated with high growth rates at both caloric levels, however, R animals fed a medium protein diet had the highest k value.

The effect of calories and protein on rates of weight gain at 10 and 20 weeks of age are graphically shown in Figure 3; means are presented in Table 3. Weight gain at 10 weeks ranged from 23.0 to 27.9 g/week in the A groups and from 16.4 to 18.4 g/week in the R groups. By 20 weeks, weight gain had decreased in the A groups and ranged from 10.4 to 12.6 g/week and in the R groups from 7.0 to 7.3 g/week. There was a significant effect of calories on weight gain at both 10 and 20 weeks ($p < 0.01$). The effect of protein on weight gain at 10 weeks was both linear and quadratic ($p < 0.01$)

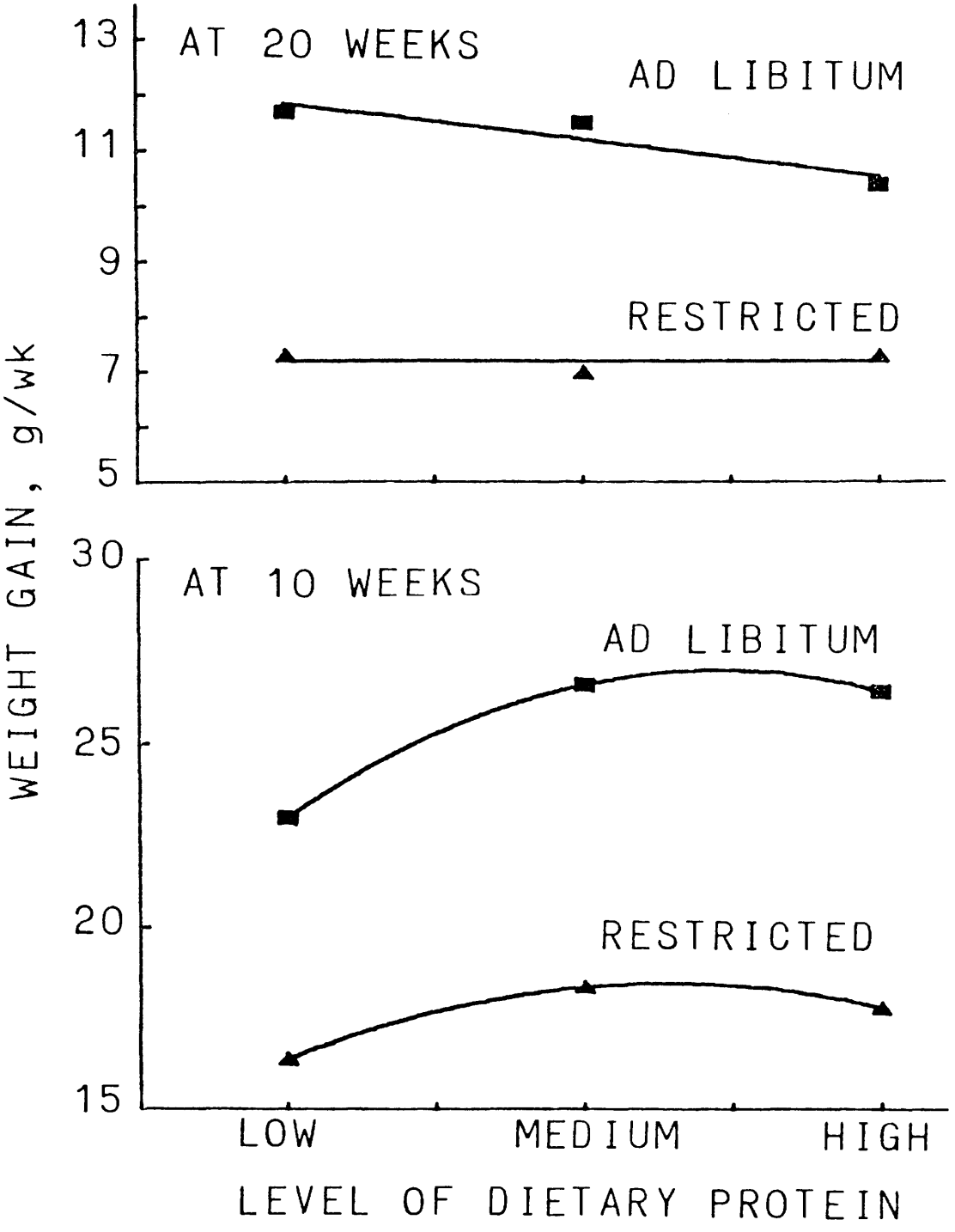

Figure 3. Influence of protein level and calories on weight gain at 10 and 20 weeks of age.

and the linear but not quadratic interaction of calories and protein was significant ($p < 0.05$). At 10 weeks of age, animals fed low levels of dietary protein on either an ad libitum or restricted basis had the lower weight gains with the effect being more pronounced in ad libitum-fed animals. At 20 weeks of age, both the effect of protein and the interaction of protein and calories were linearily related to weight gain ($p < 0.10$); weight gain was negatively associated with protein level in A groups.

Calculated growth curves and actual mean body weights during the second year of life for R and A groups consuming diets containing constant levels of low, medium, and high protein throughout life are presented in Figure 4. Actual body weights tended to lie on the predicted growth curves in the A groups and below the curves in the R groups. R and A groups fed low and medium levels of protein generally had actual body weights after 1 year of age which fell below the predicted growth curves. Figure 5 shows the calculated growth curves and actual body weights during the second year of life for animals fed ad libitum medium protein diets either decreased or increased in protein level during the first year of life. Growth curves and actual body weights for A animals fed a diet decreased from a medium to a low protein level by 1 year were higher than those for A animals fed the medium protein diet increased to a high protein level during the first year of life.

Mean feed intakes and significance levels for contrast statements of A groups at 2, 4, 6, and 12 months of age are given in Table 4 (since R groups were fed two-thirds of the amount of feed consumed by their A controls, intakes are assumed to be as fed and are not

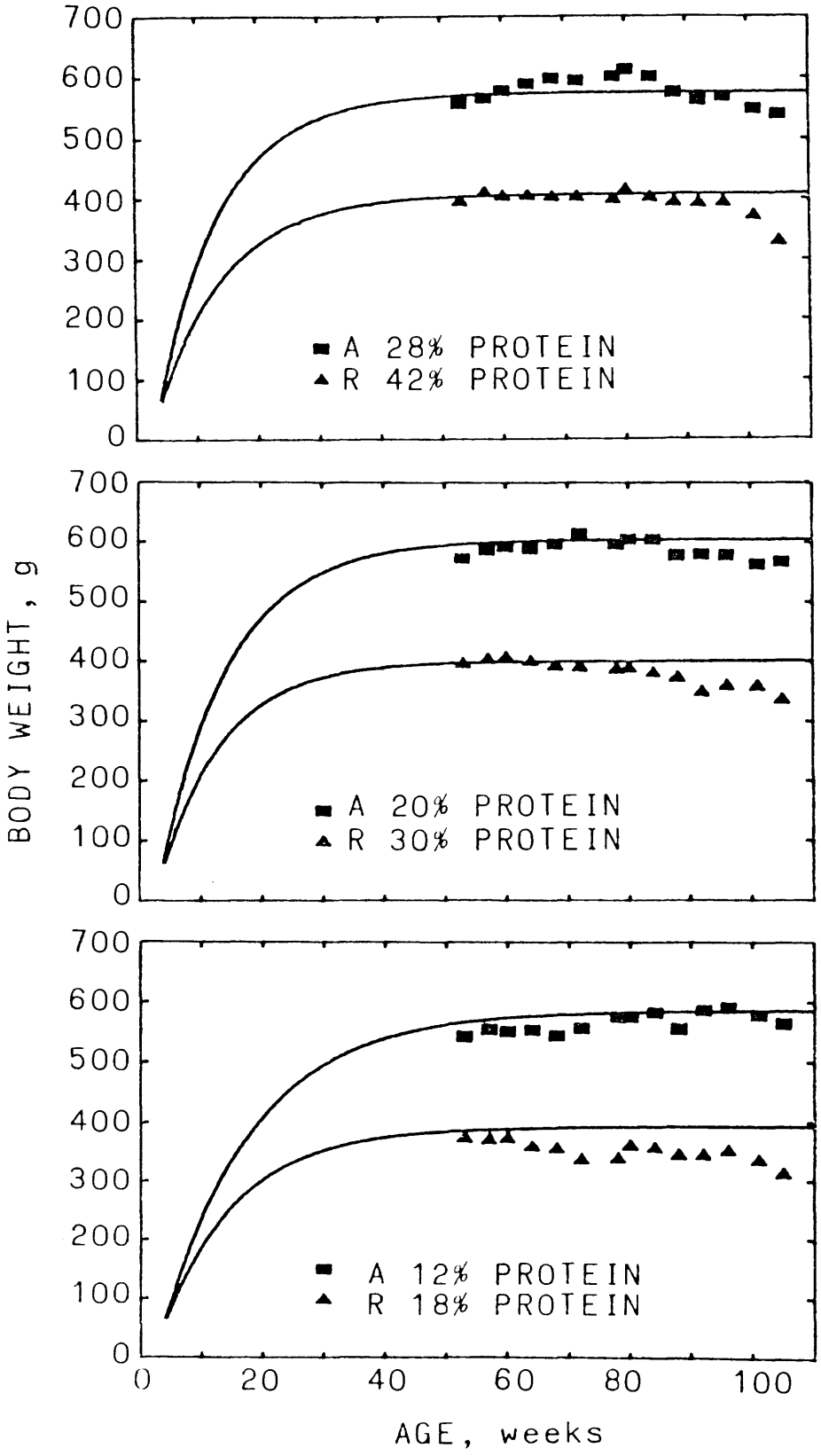

Figure 4. Influence of dietary protein level in ad libitum (A) or restricted-fed (R) rats on growth curves and mean body weights.

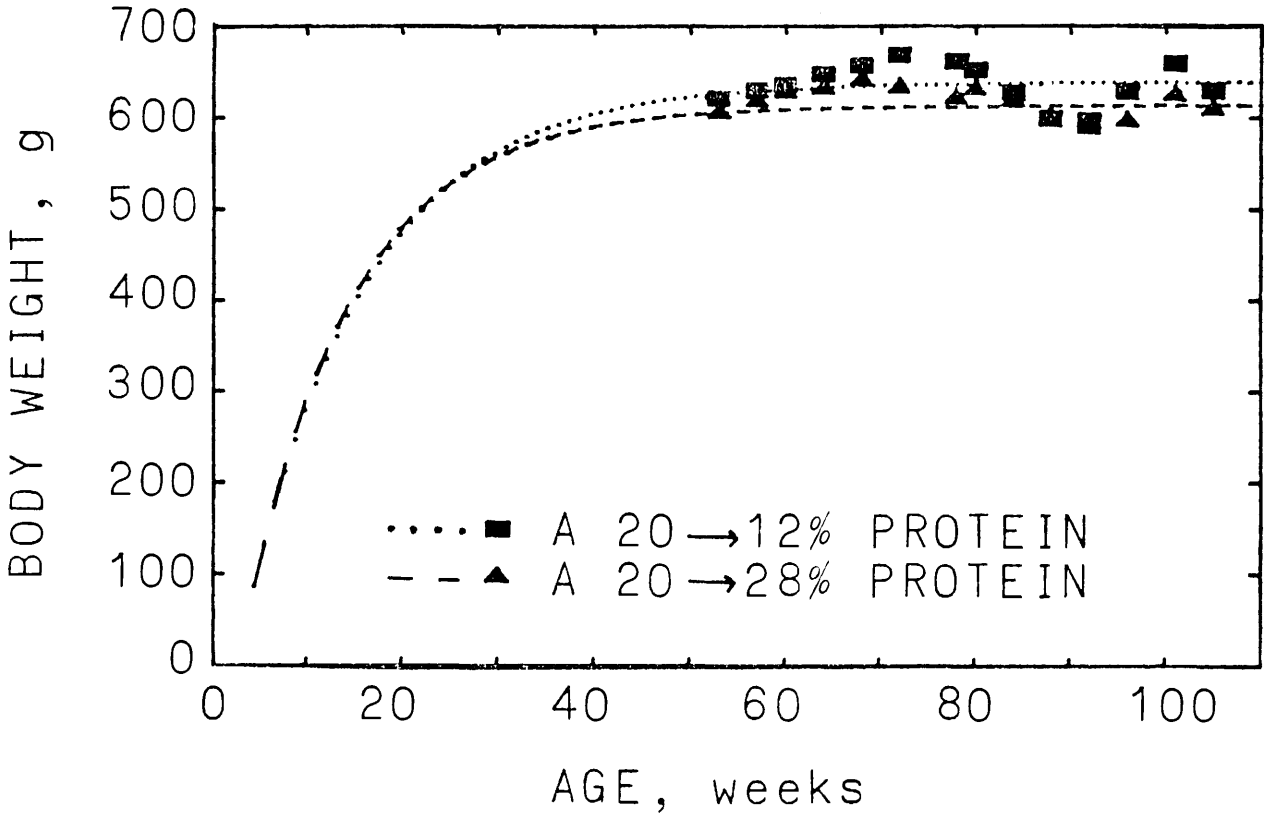

Figure 5. Influence of feeding ad libitum medium protein diets decreased and increased in protein content during the first year of life on calculated growth curves and actual mean body weights.

TABLE 4

MEANS AND SIGNIFICANCE LEVELS FOR CONTRASTS OF FEED INTAKE BY DIET AND AGE

Treatment	Months 2	4	6	12
	g/day	g/day	g/day	g/day
Percent protein				
Ad libitum				
12	17.4	18.3	17.8	17.7
20	19.4	19.7	18.6	18.3
28	18.7	18.9	18.2	18.3
20 → 12	18.3	19.7	19.2	19.2
20 → 28	19.4	19.2	18.5	18.4
SEM for column	±0.3	±0.5	±0.4	±0.4
Contrast statements		Significance[1]		
Linear for protein	***	NS	NS	NS
Quadratic for protein	***	**	NS	NS
Variable protein (20 → 12 vs 20 → 28)	**	NS	NS	NS
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	NS	NS	*	*

[1]Statistical significance for contrast statements are indicated by *($p < 0.10$), **($p < 0.05$), and ***($p < 0.01$). Contrast statements not statistically significant are indicated by NS ($p > 0.10$).

included in the table). Mean feed intakes ranged from 17.4 to 19.7 g/day and changed little from 2 to 12 months of age. There was a significant effect of protein on feed intake at 2 months (both linear and quadratic, $p < 0.01$) and 4 months (quadratic, $p < 0.05$) but thereafter dietary protein level generally had no effect on feed intake. At 2 months, there was a significant difference ($p < 0.05$) in feed intake between the 2 variable protein groups with the 20 → 28% group consuming the greater amount. After 2 months, there were no significant differences in feed intake between these 2 groups, but the 20 → 12% group always tended to consume more. A comparison of the intakes of animals fed constant levels of protein and those fed varying protein levels showed only small but significant differences at 6 and 12 months of age ($p < 0.10$).

The effect of age and diet on the in vitro transport of PAH by kidney slices is shown graphically in Figure 6. Mean values for PAH transport and kidney DNA content are presented in Table 5; the results of statistical analysis of the data are presented in Table 6. In young animals, mean PAH transport values ranged from 183.5 to 218.1 μg/g in A groups and from 171.3 to 186.4 g/g kidney slices in R groups. Comparable values for old rats ranged from 82.1 to 105.5 μg/g in A groups and from 124.5 to 139.3 μg/g in R groups. There was no significant effect of diet on PAH transport per g kidney slice in young animals but in old animals, A groups had significantly lower transport values than R groups ($p < 0.10$). Dietary protein level had no significant effect on PAH transport in young or old rats.

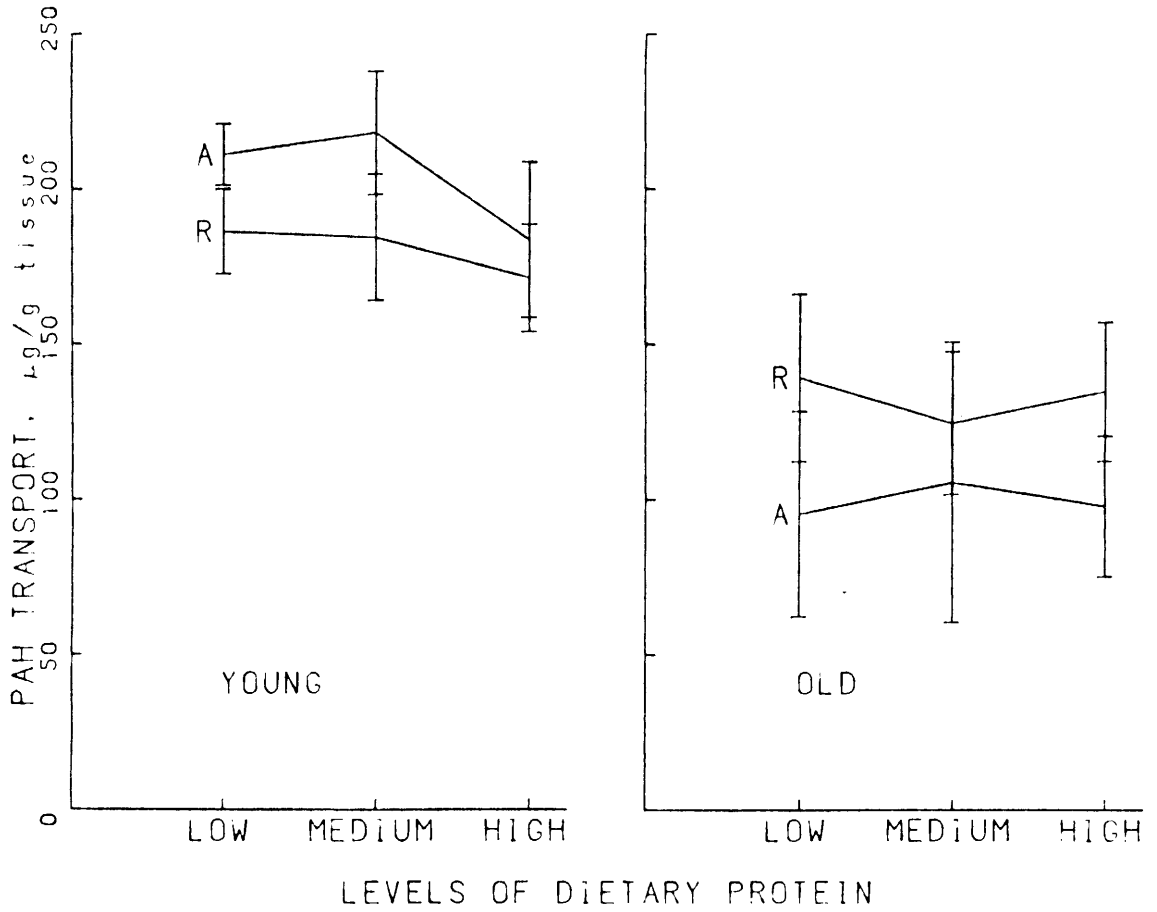

Figure 6. Influence of level of dietary protein and age on PAH transport in kidney slices of rats fed on an ad libitum (A) and restricted (R) basis. Data points are means ± SEM.

TABLE 5

MEANS FOR KIDNEY PAH TRANSPORT AND DNA
BY DIET AND AGE

Dietary group	PAH	DNA	PAH/DNA
	µg/g	mg/g	µg/mg
Young			
Ad libitum			
12[1]	211.3	4.48	47.9
20	218.1	4.64	48.7
28	183.5	4.36	42.0
20 → 12	199.6	4.62	44.1
20 → 28	188.8	4.70	42.8
Restricted			
18	186.4	4.55	41.4
30	184.5	4.51	41.9
42	171.3	4.24	40.8
SEM for column	±7.8	±0.08	±1.8
Old			
Ad libitum			
12	95.4	4.38	21.7
20	105.5	4.64	23.5
28	97.8	5.30	20.8
20 → 12	82.1	4.54	23.4
20 → 28	94.0	4.89	20.2
Restricted			
18	139.3	4.96	28.3
30	124.5	4.62	27.0
42	134.6	4.19	32.2
SEM for column	±9.9	±0.11	±2.3

[1]Percent dietary casein.

TABLE 6

SUMMARY OF STATISTICAL ANALYSES FOR KIDNEY TRANSPORT OF PAH AND DNA CONTENT

Source of variation	Significance[1] PAH	DNA	PAH/DNA
Diet	NS	NS	NS
Age	***	NS	***
Diet-age interaction	NS	NS	NS
Contrast statements			
Young			
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	NS	NS	NS
Ad libitum vs restricted	NS	NS	NS
Linear for protein	NS	NS	NS
Quadratic for protein	NS	NS	NS
Linear interaction	NS	NS	NS
Quadratic interaction	NS	NS	NS
Old			
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	NS	NS	NS
Ad libitum vs restricted	*	NS	NS
Linear for protein	NS	NS	NS
Quadratic for protein	NS	NS	NS
Linear interaction	NS	***	NS
Quadratic interaction	NS	NS	NS

[1]Statistical significance is indicated by *($p < 0.10$), **($p < 0.05$), and ***($p < 0.01$). Statements not statistically significant are indicated by NS ($p > 0.10$).

The mean DNA content of kidneys from young animals ranged from 4.36 to 4.70 mg/g in A groups and from 4.24 to 4.55 mg/g in R groups; values for old animals were 4.38 to 5.30 mg/g in A groups and 4.19 to 4.96 mg/g in R groups. Age differences in kidney DNA content were not significant. Caloric level had no significant effect on DNA values in either age group. While the overall effect of protein on kidney DNA was not significant, there was a significant linear interaction of protein and calories in old animals ($p < 0.01$); kidney DNA content was positively associated with dietary protein level in A groups but negatively associated in R groups.

In young animals, the mean kidney transport of PAH per unit DNA ranged from 42.0 to 48.7 μg/mg in A groups and from 40.8 to 41.9 μg/g in R groups. The ratio of renal PAH transport to DNA content in old rats was significantly less than that for young rats ($p < 0.01$). The ratio in old rats ranged from 20.2 to 23.5 μg/mg in A groups and from 27.0 to 32.2 μg/mg in R groups. Dietary protein level had no significant effect on the PAH to DNA ratio. R groups, however, tended to have higher ratios than A groups at 2 years but the difference was not statistically significant ($p = 0.12$).

Urinary protein excretion for groups of young and old animals fed constant levels of dietary protein on an ad libitum or restricted basis is presented in Figure 7; means for urinary protein excretion and urinary volume for all the groups are shown in Table 7. Table 8 presents the results of analyzing the urinary data statistically. In young animals, urinary protein excretion ranged from 10.83 to 40.70 mg/day in A groups and from 9.53 to 22.71 mg/day in R groups. The mean urinary protein excretion values for old groups were

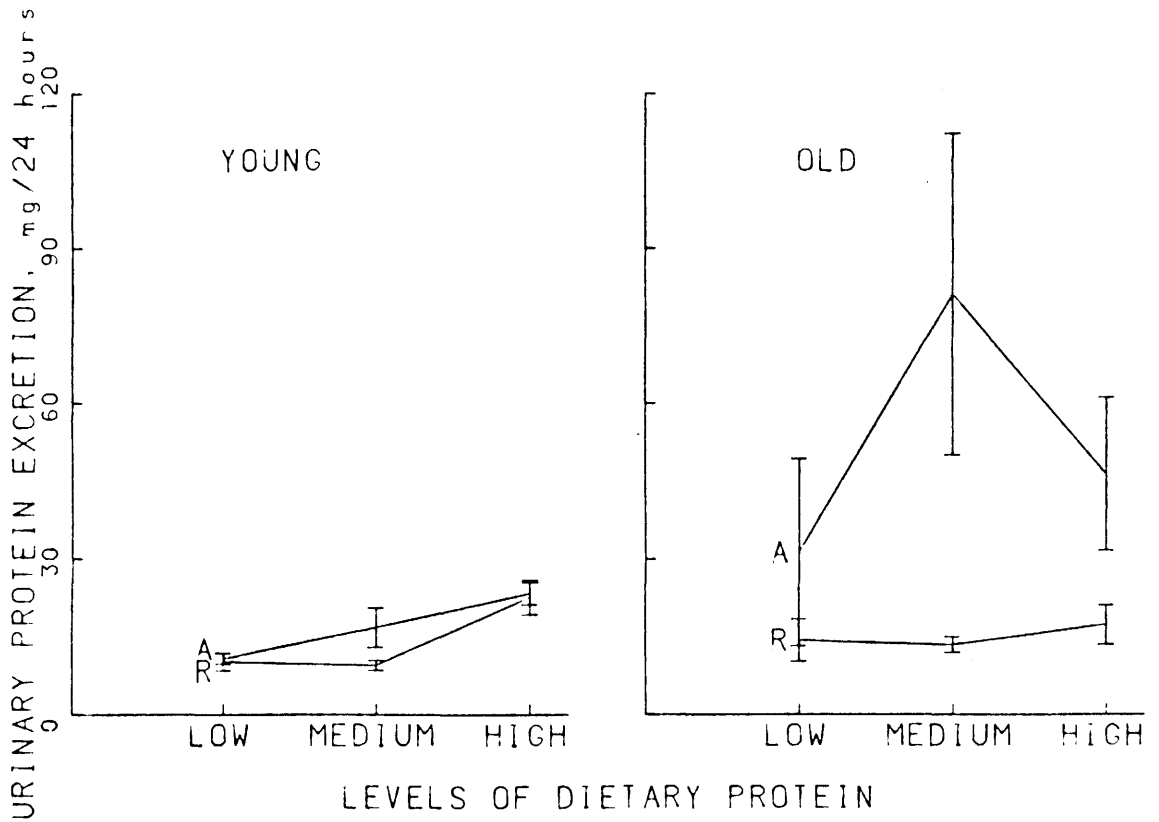

Figure 7. Influence of level of dietary protein and age on urinary protein excretion of rats fed on an ad libitum (A) or restricted (R) basis. Data points are means ± SEM.

TABLE 7

MEANS FOR URINARY PROTEIN EXCRETION AND URINE VOLUME BY DIET AND AGE

Dietary group	Urinary protein	Urine volume
	mg/day	ml/day
Young		
Ad libitum		
12[1]	10.83	2.49
20	16.85	3.10
28	23.50	4.66
20 → 12	12.67	4.13
20 → 28	40.70	4.89
Restricted		
18	10.25	5.82
30	9.53	7.28
42	22.71	8.09
SEM for column	±3.85	±0.53
Old		
Ad libitum		
12	31.30	4.18
20	81.18	6.99
28	46.18	7.99
20 → 12	25.74	3.28
20 → 28	71.44	13.00
Restricted		
18	14.40	9.95
30	13.48	9.44
42	17.34	12.94
SEM for column	±4.09	±0.56

[1]Percent dietary casein.

TABLE 8

SUMMARY OF STATISTICAL ANALYSES FOR URINARY PROTEIN EXCRETION AND URINE VOLUME

Source of variation	Significance[1] Urinary protein excretion	Urine volume
Diet	***	***
Age	***	***
Diet-age interaction	*	NS
Contrast statements		
Young		
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	NS	NS
Ad libitum vs restricted	NS	***
Linear for protein	NS	NS
Quadratic for protein	NS	NS
Linear interaction	NS	NS
Quadratic interaction	NS	NS
Old		
Constant protein A (12, 20, 28%) vs variable protein A (20 → 12, 20 → 28%)	NS	NS
Ad libitum vs restricted	***	***
Linear for protein	NS	**
Quadratic for protein	**	NS
Linear interaction	NS	NS
Quadratic interaction	**	NS

[1]Statistical significance is indicated by *($p < 0.10$), **($p < 0.05$), and ***($p < 0.01$). Statements not statistically significant are indicated by NS ($p > 0.10$).

significantly greater than those for young groups ($p < 0.01$) and ranged from 25.74 to 81.18 mg/day in A groups and from 13.48 to 17.34 mg/day in R groups. Diet had no significant effect on urinary protein excretion of young animals, but in old animals, protein excretion was significantly greater in A than in R groups ($p < 0.01$). There was also a significant quadratic effect of protein and a protein-calorie interaction in old animals ($p < 0.05$); high excretion rates were generally associated with high dietary protein levels although the highest mean protein excretion value was found at the medium dietary protein level of the A groups.

The mean urine volumes of young rats ranged from 2.49 to 4.89 ml/day in A groups and from 5.82 to 8.09 ml/day in R groups; values for old animals were 3.28 to 13.00 ml/day in A groups and 9.95 to 12.94 ml/day in R groups. Old rats as compared to young excreted significantly larger volumes of urine ($p < 0.01$). Urine volume per day was significantly greater in R than in A animals of both age groups ($p < 0.01$) and was linear for dietary protein level in old animals ($p < 0.05$), i.e., high levels of dietary protein were associated with large urinary volumes.

Table 9 shows the correlation coefficients between PAH transport (μg/g kidney) and urinary protein excretion (mg/day) by dietary group in young and old animals. There were no significant correlations between PAH transport and urinary protein in any of the dietary groups at 1 year of age. Likewise, in old animals fed restricted diets or low protein diets ad libitum, the two renal function measurements were not significantly correlated. However, in the other 4 old

TABLE 9

ESTIMATED CORRELATIONS BETWEEN RENAL PAH TRANSPORT
URINARY PROTEIN EXCRETION

Dietary group	Correlation coefficient[1]
Young	
Ad libitum	
12[2]	0.711
20	0.532
28	-0.132
20 → 12	0.003
20 → 28	-0.498
Restricted	
18	0.537
30	0.474
42	-0.272
Old	
Ad libitum	
12	-0.850
20	-0.920**
28	-0.899***
20 → 12	-0.974***
20 → 28	-0.909**
Restricted	
18	-0.185
30	0.284
42	-0.419

[1]Correlation coefficients followed by superscripts are statistically significant (*, $p < 0.10$; **, $p < 0.05$; and ***, $p < 0.01$).

[2]Percent dietary casein.

A groups, protein excretion and PAH transport were significantly negatively correlated ($p < 0.05$).

The percent survival for all dietary groups at 1, 1.5, and 2 years of age and the significances of contrasts are presented in Table 10. There was no effect of diet on percent survival at 1 year but at 1.5 years, the effect of caloric level was significant ($p < 0.10$). At time of sacrifice (2 years), percent survival ranged from 19.4 to 33.3% in A groups and from 30.6 to 61.1% in R groups and the difference in percent survival between R and A groups was statistically significant ($p < 0.01$). There was also a significant linear ($p < 0.05$) and quadratic ($p < 0.10$) effect of protein but no significant interaction of calories and protein. Low dietary protein levels were associated with low percent survivals in both caloric groups (see Figure 8). However, A animals provided medium and high protein diets had similar survival rates while the highest percent survival was in R animals fed a medium protein diet.

TABLE 10

MEANS AND SIGNIFICANCE LEVELS FOR CONTRASTS OF PERCENT SURVIVAL BY DIET AT 1, 1.5 AND 2 YEARS

Treatment	Percent survival 1 yr	1.5 yr	2 yr
Percent protein			
Ad libitum			
12	91.7	63.9	19.4
20	86.1	72.2	30.6
28	94.4	66.7	33.3
20 → 12	91.7	77.8	25.0
20 → 28	86.1	72.2	30.6
Restricted			
18	100.0	66.7	30.6
30	94.4	86.1	61.1
42	94.4	80.6	52.8
Contrast statements		Significance[1]	
Ad libitum vs restricted	NS	*	***
Linear for protein	NS	NS	**
Quadratic for protein	NS	NS	*
Linear interaction	NS	NS	NS
Quadratic interaction	NS	NS	NS

[1]Statistical significance for contrast statements are indicated by *($p < 0.10$), **($p < 0.05$), and ***($p < 0.01$). Contrast statements not statistically significant are indicated by NS ($p > 0.10$).

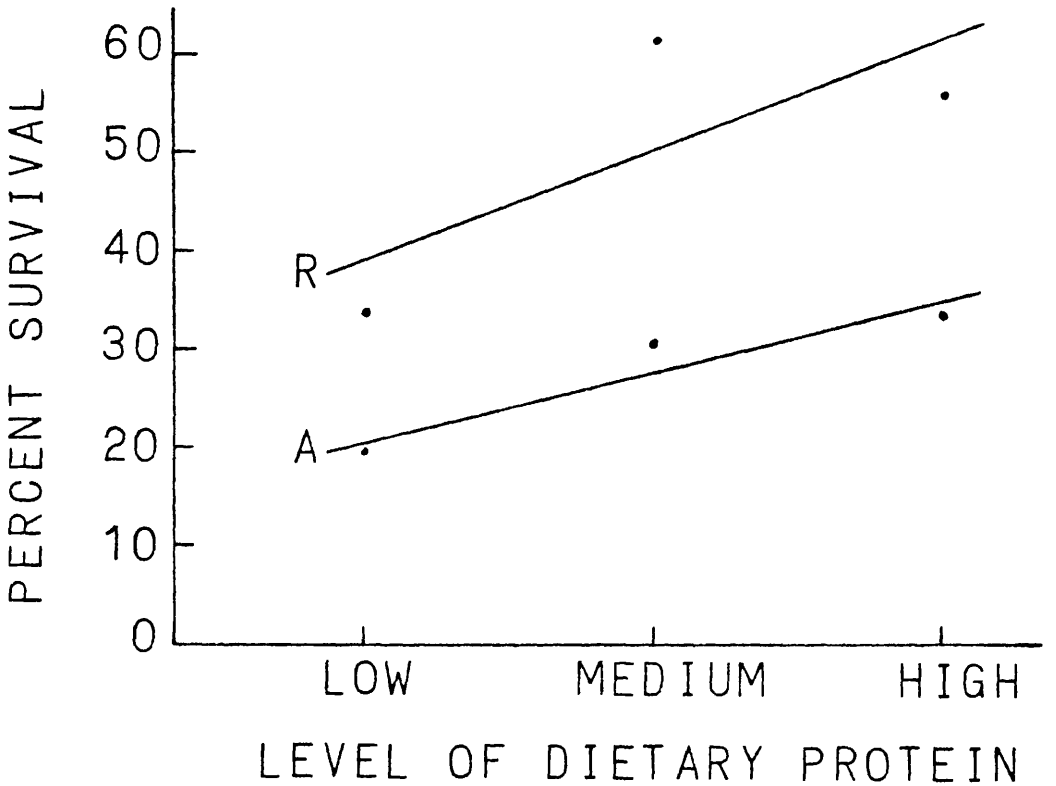

Figure 8. Influence of dietary protein level on percent survival of ad libitum (A) and restricted-fed (R) rats at 2 years of age.

CHAPTER V

DISCUSSION

The calculated mature body weights of restricted-fed animals (R) were approximately two-thirds of those for ad libitum-fed (A) rats as has been reported by other investigators in animals fed two-thirds of ad libitum feed intake (6) and in those intermittently-fed (12). While Berg (6) reported that the shapes of the growth curves for restricted rats were similar to those for unrestricted rats, the present study showed a significant difference in the calculated growth rate (k) in R as compared to A groups. This difference in growth rate between A and R groups was primarily due to the lower growth rate (k) in animals fed ad libitum low protein diets which did not meet the protein requirements for growth (13.3% casein) but exceeded that for weight maintenance (4.4% casein) (57). During the early stages of the growing period, low dietary protein intakes were associated with the smallest weight gains in either A or R groups but the greatest protein effect was exhibited at the higher caloric level, e.g., in ad libitum-fed animals. During the later stages of the growing period, the effect of dietary protein level on weight gain tended to be reversed, i.e., low dietary protein levels were associated with large weight gains especially in ad libitum-fed groups. Calculated mature body weights and actual body weights were similar in animals fed different dietary protein levels but the same amount of calories. These findings are in agreement with those of Nakagawa et al. (18) and Ross et al. (58) who found

that rats fed low protein diets grew at a slower rate but reached a similar mature body weight as those fed normal protein diets. In the present study, the greater magnitude of the effect of protein on growth rate and rates of weight gain at 10 and 20 weeks in A groups than in R groups would indicate that additional calories were needed to optimize the effect of protein on growth in R animals. Since the consumption of medium or high amounts of protein in R groups resulted in only slightly different values for growth variables from those for animals fed low protein diets, it would appear that caloric intake was the main limiting factor in the growth of R rats provided low protein diets.

The high mature body weights but similar k values of A animals fed varying levels of protein during the first year of life as compared to those fed a constant dietary protein level throughout life were probably due to the significantly greater feed intakes of the animals fed variable protein diets. Animals fed the medium protein diet which was gradually reduced in protein content had the highest feed intakes after 6 months of age, the greatest weight gain at 20 weeks, the lowest growth rate (k) with the exception of animals fed a low protein diet ad libitum throughout life, and the largest mature body weight. It is likely that these animals had a lean body mass similar to other dietary groups but accumulated more body fat. The relatively low feed intake of animals fed a low protein diet ad libitum throughout life is consistent with reports of others who found a 10% reduction in feed intake of animals fed low as compared to normal dietary protein levels (13,58).

The mean DNA value of 4.62 mg/g renal tissue in the present

study was similar to that of 4.93 mg/g reported by Oppenheimer et al. (59) in young Sprague-Dawley rats but greater than the 3.36 mg DNA/g kidney of adult female Fu-albino rats found by Kestler (60). While Beauchene et al. (34) showed a significant increase with age in renal DNA content from 3.07 in young to 3.58 mg/g in old Wistar rats, there was no effect of age on DNA values in the present study. Although there was no significant effect of protein in young or old animals and no significant protein-calorie interaction in young animals on renal DNA content, there was a significant linear interaction of protein and calories in old animals such that high protein intakes in A groups and low protein intakes in R groups were associated with high levels of kidney DNA. The high DNA content of kidneys from old restricted animals fed low protein diets is consistent with the results reported by Barrows and Kokkonen (45) in young mice. If high levels of DNA per unit wet weight indicates small cell size, these results suggest that restricted animals fed low protein diets have small kidney cells.

The findings of an increased urinary protein excretion with age in A groups is in agreement with the work reported by others (12,32-34,37). Age did not significantly affect proteinuria in R groups, thus indicating the beneficial effect of caloric restriction on this parameter as was reported by Tucker et al. (12). Although there was a direct statistical association between dietary protein level and proteinuria in old rats especially those ad libitum-fed, the highest protein excretion rate was in animals fed ad libitum medium protein diets. While caloric restriction had the greater effect in delaying the age-associated increase in urinary protein

excretion, low dietary protein levels also had a beneficial effect on slowing the rate of the kidney aging process.

The decrease in transport of PAH by kidney slices in old as compared to young ad libitum-fed animals agrees with the work reported by others (12,31,50). While caloric restriction had no significant effect on PAH transport in young animals, old rats fed restricted diets had significantly higher values for PAH transport per unit wet weight than those ad libitum-fed but not when expressed per unit DNA. If renal DNA content is indicative of tubule cell number, it would appear that renal tubules of old A animals may function as well as those of old R animals. However, the difference in transport values per unit DNA between the 2 caloric levels approached significance ($p \approx 0.12$) and the results from all other kidney function tests indicate that kidneys of old restricted rats functioned better than those from ad libitum-fed rats.

While diet had a greater effect on urinary protein excretion than on PAH transport, there was a significant correlation (as urinary protein increased, PAH transport decreased) between these 2 parameters in most old ad libitum-fed groups, and thus either variable could be used as an indication of renal function. The lack of a significant correlation between proteinuria and PAH transport for young rats fed ad libitum, young or old rats fed on a restricted basis, and old rats fed a low protein diet ad libitum was probably related to the low levels of urinary protein excreted by these dietary groups. The results of renal function tests indicate that the level of caloric restriction used in this study delayed the age-associated decline in renal function and that protein restriction had a beneficial

effect but not of the same magnitude as the reduction of calories. It appears that the animals' renal performance was more sensitive to the levels of caloric intake than to the levels of dietary protein employed in this study.

If urine volume is a reflection of water intake, the increase in urine volume from 5.36 in young to 8.77 ml/day in old rats is consistent with the increased water intake with age in rats reported by Jakubczak (61) and Peng et al. (62). It is likely that this increase in urine volume with age is related to a decreased ability of old rats to concentrate urine (30). While Jakubczak (61) found water intake to be directly proportional to feed intake, the present study showed a significantly greater urine volume in restricted as compared to ad libitum-fed animals. The large urine volumes of R groups would appear to indicate a detrimental effect of caloric restriction on renal function. This is inconsistent, however, with the effect of caloric restriction on other renal measurements and therefore is probably related to the dietary restriction itself, i.e., the rats may have consumed more water in an attempt to satisfy their appetite for food.

Old A or R rats fed high protein diets excreted larger volumes of urine than those fed low protein diets. This finding is consistent with the increased osmotic load associated with high protein diets, i.e., conversion of protein to urea which is excreted by the kidney. In addition, the increased urinary protein excretion associated with high levels of dietary protein in old animals would have a direct effect on the osmotic load.

Since the biochemical measurements required sacrifice of the animals at 2 years of age, longevity analysis was based on the percent survival of rats at various ages rather than mean life span. The increase in the percent survival of rats fed calorically restricted diets as compared to those fed ad libitum is consistent with the findings reported by other investigators (4-11,20,23,24).[1] While in the present study R and A animals consumed relatively the same quantity of protein, in most studies the reduction in caloric intake was accompanied by a decrease in protein intake (4-11,20).[1] Although caloric intake had the greater effect on survival, dietary protein level also significantly influenced survival rate. The increase in percent survival with increasing dietary protein levels in both caloric groups is inconsistent with the work of Leto et al. (13,14), Miller and Payne (15), and Goodrick (16) but is in agreement with that reported by Ross and Bras (20,24). While the overall effect of protein on percent survival was linear, survival also exhibited a quadratic effect such that the percent survival of rats fed high protein diets at both caloric levels was similar to or less than that for rats fed medium protein diets. This would indicate that while higher dietary protein levels were associated with increased percent survival, there was some optimum level of protein intake for maximizing longevity and that the medium protein diets of the present study were approaching this level.

Since R groups had significantly lower body weights and rates of weight gain and better renal function and percent survival at 2

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[1]Beauchene et al. (1979) The effect of feed restriction on body composition and longevity of rats.

years than A animals, it would appear that a decrease in growth has a beneficial effect on kidney function and the life span of animals. The agreement between renal function measurments and percent survival on the effect of caloric restriction on the aging process and the inconsistency for the effect of dietary protein level indicate that caloric restriction has the greater nutritional effect on delaying the aging process. The positive association between the level of protein in the diet and urinary protein excretion and between dietary protein level and percent survival could indicate that the increase in urinary protein excretion with dietary protein level was related to factors other than those which affect longevity. While low dietary protein levels were beneficial to a specific organ, i.e., the kidney, other biological systems must respond favorably to higher levels of dietary protein and therefore the survival of these animals was positively affected by medium or moderately high levels of protein in the diet.

It can be concluded that caloric restriction reduced mature body weight, delayed the age-associated decline in renal function, and increased the survival of rats and therefore slowed the rate of the aging process. While dietary protein level did not have as great an effect on these parameters as caloric intake, low levels of protein reduced the rate of growth, generally improved renal function but decreased survival. It appears that while high levels of dietary protein adversely affected the function of a specific biological system, i.e., the kidney, medium or moderately high protein diets must be beneficial for other systems in these animals and therefore favorably influence survival.

CHAPTER VI

SUMMARY

The effects of caloric restriction without concomitant protein restriction and protein restriction with and without caloric restriction on growth, renal function, and survival of male rats were investigated. In addition, the effect of gradually changing the level of protein in the diet during growth and development without reducing caloric intake on these parameters was studied.

Ad libitum-fed (A) rats had significantly greater rates of weight gain during growth and higher mature body weights than restricted-fed (R) animals but significantly lower growth rates (k). While dietary protein level had no significant effect on mature body weight, it did affect the rate at which mature body weight was attained. The overall effect of protein on growth rate (k) was linear. High dietary protein levels were generally associated with high k values at both caloric levels, however, R animals fed a medium protein diet had the highest k value.

The effect of protein on weight gain at 10 weeks of age was both linear and quadratic and the protein-calories interaction was linear; low dietary protein levels were associated with the smaller weight gains especially in A groups. At 20 weeks of age, both the effect of protein and the interaction of protein and calories were linearily related to weight gain such that animals fed low protein diets ad libitum gained more weight than those fed higher protein diets. Dietary protein level had a greater effect on the growth of

rats fed ad libitum than on that of R groups. Animals fed ad libitum varying levels of protein during the first year of life had higher mature body weights and rates of weight gain at 10 and 20 weeks of age than those fed a constant level of protein and generally consumed more diet. Animals fed a constant low level of protein tended to consume smaller quantities of diet than those fed higher dietary protein levels.

The transport of PAH by kidney slices was significantly greater in young than in old animals and was significantly higher in old R groups than in old A groups when expressed per unit wet weight. R groups tended to have higher ratios of kidney PAH transport to DNA content than A groups at 2 years but the difference was not statistically significant. Dietary protein level had no significant effect on PAH transport. There was no significant effect of either age or diet on kidney DNA content except for a linear interaction of protein and calories in old animals; DNA values were positively associated with dietary protein level in old ad libitum-fed groups but negatively associated in those fed restricted diets.

Urinary protein excretion did not increase with age in R groups but was significantly greater in old than in young animals in A groups. There was a significant quadratic effect of protein and protein-calorie interaction in old animals on urinary protein; proteinuria generally increased with dietary protein level although the highest mean protein excretion value was found at the medium dietary protein level in the A groups. Both young and old R animals excreted significantly greater volumes of urine than those in A groups.

The effect of dietary protein was linear in old animals; high levels of dietary protein were associated with large urine volumes.

There were no significant correlations between PAH transport and urinary protein excretion in any of the dietary groups at one year of age. At 2 years of age, however, the two renal function measurements were significantly correlated (as urinary protein increased, PAH transport decreased) in most ad libitum-fed groups. While caloric restriction had the greatest effect on slowing the renal aging process, protein restriction also was beneficial in delaying the age-associated decline in renal function.

The percent survival of R groups was significantly greater than that for A groups at 1.5 and 2 years of age. At 2 years, there was a significant linear and quadratic effect of protein. While survival rate was directly associated with dietary protein level, the percent survival of rats fed high protein diets at both caloric levels was less than or similar to that for R and A animals fed medium protein diets indicating there was an optimum level of protein intake which enhanced survival.

In general, the hypotheses that caloric restriction decreased mature body weight and slowed the aging process as indicated by an improvement in renal function and an increase in survival of old animals fed restricted diets throughout life were supported. The effect of protein restriction was not of the same magnitude as caloric restriction and while a low protein diet reduced the rate of growth and was beneficial to renal function, it adversely affected survival. It appears that biological systems other than the kidney must have responded favorably to higher dietary protein levels and

therefore survival was enhanced in rats fed medium or moderately high protein diets.

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VITA

Teresa Ann Davis was born in Middlesboro, Kentucky on March 23, 1955. She attended elementary school in Claiborne County, Tennessee and was graduated from Powell High School, Powell, Tennessee in June 1971. The following September she entered The University of Tennessee, Knoxville, and in August 1975, she received her Bachelor of Science degree in Home Economics Education with high honors. She entered the Graduate School of The University of Tennessee in the Fall of 1975 and in August 1976, she received the Master of Science degree with a major in Nutrition. The following quarter she began work toward the Ph.D. degree. During her graduate program, she received a General Foods Fellowship and served as a graduate assistant, graduate teaching assistant, and graduate research assistant in the Department of Food Science, Nutrition, and Food Systems Administration. In August 1980, she received the Doctor of Philosophy degree with a major in Home Economics.