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The Effect of Protein and Caloric Restriction and Age on the Rate of Liver Protein Synthesis of Male Rats

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To the Graduate Council:

I am submitting herewith a dissertation written by Connie Watkins Bales entitled "The Effect of Protein and Caloric Restriction and Age on the Rate of Liver Protein Synthesis 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, Marjorie P. Penfield

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
To the Graduate Council:

I am submitting herewith a dissertation written by Connie Watkins Bales entitled "The Effect of Protein and Caloric Restriction and Age on the Rate of Liver Protein Synthesis of Male Rats." I have examined the final 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 Home Economics.

We have read this dissertation and recommend its acceptance:

Roy E. Beauchene, Major Professor

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research
THE EFFECT OF PROTEIN AND CALORIC RESTRICTION AND AGE ON THE RATE OF LIVER PROTEIN SYNTHESIS OF MALE RATS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Connie Watkins Bales
August 1981
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ABSTRACT

The liver protein synthesis rate and protein content were studied in male Wistar rats fed low-, medium-, and high-protein diets at 2 calorie levels for 1 (12 animals per dietary group) or 2 (36 animals per dietary group) years. Ad libitum-fed (A) groups were offered semisynthetic diets containing 12, 20, or 28% casein. Restricted-fed (R) rats fed diets containing 18, 30, or 42% casein were provided two-thirds of the mean quantity of diet consumed by A groups and thus consumed the same amount of protein but one-third fewer calories than their ad libitum-fed controls.

The fractional rate of liver protein synthesis was determined by the method of continuous dietary infusion of $[^{14}\text{C}]$tyrosine. The rate of rise in the specific activity of free tyrosine (rate constant) was approximated for each rat using the rate of rise of $^{14}\text{C}$ radioactivity in the expired CO$_2$. The rate constant and the specific radioactivity of the free and protein-bound liver tyrosine were utilized to calculate the fractional rate of protein synthesis.

Restricted-fed rats had growth curves similar in shape but at a lower level than those for ad libitum-fed rats. Mean daily feed intake of A groups showed little variation after 2 months of age (R groups were fed two-thirds of the amount consumed by their ad libitum-fed controls).

Percent survival was increased ($p < 0.003$) by caloric restriction. Although caloric restriction resulted in a greater effect on lifespan, an effect of dietary protein intake was also demonstrated. Low dietary
protein intake was associated with a low percent survival in both caloric groups.

With age, there was a reduction (p < 0.05) in liver protein content. Protein restriction produced a linear decrease (p < 0.03) in liver protein content in 1-year animals only. Caloric restriction was associated with a dramatic increase (p < 0.0001) in liver protein content in both young (1-year) and old (2-year) animals.

The liver protein synthetic rate increased (14%) with age (p = 0.02). In old animals, dietary protein restriction produced a linear increase (p < 0.01) in the rate of liver protein synthesis of both ad libitum- and restricted-fed groups. Young restricted-fed groups exhibited a similar tendency. A dramatic reduction (p < 0.0001) in the liver protein synthetic rate as a result of caloric restriction was observed in 2-year animals and the same trend was noted in young restricted-fed groups. Rates of protein synthesis exhibited an interaction (p < 0.02) between protein intake and caloric level, that is, R groups showed a linear increase in liver protein synthesis with protein restriction while A groups showed little change.

Caloric restriction delayed the age-associated increase in liver protein synthesis and increased percent survival, while protein restriction was associated with an increase in the liver protein synthetic rate and a reduction in percent survival. These findings support the hypothesis that the mechanism by which caloric restriction increases longevity is through decreased use of the genetic code for protein synthesis that reduces genetic imperfections that may occur during aging.
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CHAPTER I

INTRODUCTION

Aging is accompanied by a dramatic decline in the functional capacity of the individual as well as the functional capacities of the individual's organs and tissues (1). Because of the importance of protein for the maintenance and functioning of cells and tissues, it has been suggested that age-related alterations in protein synthesis may be among the major causes of aging (2). It is not known if these age-associated changes are strictly programmed or if they occur because genetic apparatus disturbances are increasing with age (3).

It has been well documented (4) that feed restriction can prolong the life span of laboratory animals. However, the mechanisms involved in the prolongation of the life spans of animals by feed restriction have not been clearly elucidated. The extension of life span is partially due to a delay in the onset and incidence of age-related diseases. However, evidence also suggests that feed restriction may modify and postpone at least some of the innate changes of aging (5). It has been proposed that the mechanism by which dietary restriction increases longevity is through decreased use of the genetic code for protein synthesis, which reduces genetic imperfections that may occur during aging (6, 7). However, the effect of aging on rates of protein synthesis has not been delineated.

Investigations of the effects of protein and caloric restriction on protein synthesis in mammalian tissues have produced conflicting
results. The liver has been studied by many workers because of its high protein synthetic activity (2). Some groups of workers have reported a decreased rate of hepatic protein synthesis in rats deprived of protein or starved for short time periods, while others have reported an increased rate or no change (8-12).

It was the purpose of the present study to elucidate the effects of protein and caloric restriction and age on the rate of protein synthesis by the liver and its protein content. The procedure utilized to study protein synthesis was that of continuous infusion of a labeled amino acid (10). Infusion was accomplished by incorporating the labeled amino acid \([^{14}\text{C}]\text{tyrosine}\) into experimental diets and feeding the diets to rats over a 6-hour period. The rate \((\lambda_f)\) at which the specific activity of the precursor free amino acid pool reached plateau level was estimated from the rate of rise of radioactivity in the expired \(\text{CO}_2\) (13). The synthetic rate of the liver protein was calculated using the rate constant \((\lambda_f)\) and the specific radioactivity of the free and protein-bound liver tyrosine.

It was hypothesized that protein restriction would produce a decrease in liver protein content and its synthetic rate. Additionally, it was hypothesized that caloric restriction would decrease the fractional rate of protein synthesis. It was further proposed that the rate of liver protein synthesis would increase with age.
CHAPTER II

REVIEW OF LITERATURE

A. THE EFFECTS OF CALORIC AND PROTEIN RESTRICTION ON GROWTH AND LONGEVITY

Total Dietary Restriction

It has been said that the most effective experimental method of extending the life span of mammalian organisms is through dietary manipulation (4). The first scientific evidence that the life span of vertebrates (trout) could be extended by dietary restriction was reported by McCay and co-workers in 1929 (14). Later McCay et al. (15, 16) demonstrated that simple restriction of caloric intake in rats consuming diets otherwise nutritionally adequate led to growth retardation and the prolongation of life span. Berg and Simms (17) confirmed these findings, reporting that restriction of a standard laboratory diet throughout life increased life span by about 25% and delayed the onset of tumors and cardiac, renal, and vascular lesions. The classic work of McCay and co-workers has been repeatedly confirmed by others (18-21). In addition, the increased life span associated with feed restriction has been observed in mice (22) and in hamsters (23). Recently, Masoro et al. (24) reported the life span extending effects of feed restriction in specific pathogen-free rats, which are characteristically longer lived than conventional rats.

It was once thought that the prolongation of life span associated with caloric restriction could be achieved only when animals were restricted early in life and thus growth and maturation were retarded.
More recent research has demonstrated that restricting feed intake at a later stage in life, thus allowing normal growth and development, still may provide the beneficial effects of dietary manipulation on life span. In a study of the effects of a 50% dietary restriction during the first and second years of life in hamsters, mice, and rats, Stuchlíková et al. (23) showed that animals fed ad libitum had the lowest survival rate while animals restricted during the first year of life and fed ad libitum thereafter achieved the longest life span. Rats restricted by the method of intermittent feeding during the first and/or second year of life showed a lengthening of life span over that of ad libitum-fed animals in a recent study in our laboratory. Thus it is not necessary to curtail growth and maturation to produce prolongation of life in rats, although the effects of restriction depend upon the severity of the restriction and the age at which it is introduced (25, 26).

**Dietary Protein Restriction**

That the composition of the diet may modify the effects of total feed restriction on the life span has been reported by several groups. Because of the metabolic and nutritional interrelationships between dietary energy and protein, the effect of dietary protein intake on life span has been investigated. Miller and Payne (27) found that female hooded rats fed a high-protein stock diet until 120 days of age and then switched to a low-protein diet had longer life spans than did animals fed the stock diet throughout life. Nakagawa et al. (28) failed

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to show an effect of dietary protein concentrations of 10, 18, 27, and 36% on longevity of female Sprague-Dawley rats. Leto et al. (29, 30) reported that feeding female C57BL/6J mice a 4% protein diet ad libitum extended life span as compared to that of animals fed 26% protein ad libitum. Goodrick (31) fed various strains of mice diets containing low amounts of protein (4% casein) or normal amounts (26% casein) and found that the prolonged growth period of mice fed a diet low in protein (4% casein) was associated with an increase in life span. Stoltzner (32) recently reported that Balb/c male mice fed 4% protein diets had marginally prolonged life spans when compared with controls fed 24% protein diets. In contrast, Ross and Bras (33) reported a positive correlation between the amount of protein consumed and life expectancy for rats fed diets ad libitum or in restricted amounts. It can be concluded that the effect of dietary protein on life span of laboratory animals has not been clearly established.

B. PROCEDURES FOR MEASUREMENT OF TISSUE PROTEIN SYNTHESIS

The measurement of protein synthetic rate is complex, and more so in liver than in many other tissues because the liver is synthesizing not only hepatic proteins but several proteins to be secreted into the plasma. In early studies protein synthetic rates were derived from the extent of incorporation of a radioactively labeled amino acid into protein following a single injection ("pulse dose") of that amino acid. This method does not provide a true estimate of the rate of protein synthesis because of the difficulty in determining the specific activity of the labeled precursor amino acid and its variation with time (34).
Arias et al. (35) introduced a double isotope method for studying protein turnover, which was a modification of the standard pulse-labeling technique. One isotopic form ($^{14}$C) of amino acid was administered initially and allowed to decay for a definite period of time. A second isotopic form ($^3$H) of the same amino acid was then administered and the animal was sacrificed shortly thereafter. The ratio of $^3$H:$^{14}$C radioactivity in a protein isolated from the animal provided a relative indication of its rate of degradation (36). This technique was intended for comparison of the turnover of several proteins having the same intracellular origin (37). McNurlan et al. (38) have described another method for measuring rates of liver protein synthesis. A single injection of a large dose of labeled amino acid was utilized in order to saturate all amino acid pools to the same specific radioactivity so that the problem of measuring the specific radioactivity of the free amino acid at the site of protein synthesis was minimized.

In 1968, Waterlow and Stephen (10) introduced the technique of continuous infusion of a radioactively labeled amino acid to estimate rates of tissue protein synthesis. The technique has been refined and utilized by others (11, 13, 39). Continuous infusion of a labeled amino acid allows the measurement of the specific activity of the precursor amino acid pool. The specific activity of the infused amino acid reaches a plateau after a period of infusion. Subsequently, the incorporation of labeled amino acid into protein proceeds in a linear manner (34). Thus, when the approximate rate at which the amino acid reaches its plateau level is determined and the specific radioactivity of the tissue free and protein-bound amino acid is measured, the liver synthetic rate can be ascertained. McNurlan et al. (38) have indicated that continuous
 infusion of liver with a labeled amino acid measures the rate of synthesis of intracellular protein and not that of secreted protein.

C. THE EFFECTS OF AGE AND PROTEIN AND CALORIC RESTRICTION ON LIVER PROTEIN CONTENT

There is limited information available concerning the effect of age on the liver protein content of rats. It has been reported by Beauchene et al. (40) that there is little difference in liver protein content of adult (10-14 month) versus senescent (24-31 month) rats. Studies by Barrows et al. (41, 42) have shown no difference in liver protein nitrogen in young (12-14 month) versus old (24-27 month) McCollum rats of both sexes. However, more information is needed before the effect of age on liver protein content can be adequately described.

It has been well documented, at least in short-term investigations, that liver protein content is decreased by protein restriction. Early work by Hill et al. (43) demonstrated that protein restriction, with and without caloric restriction, resulted in a decrease in the liver protein content of Sprague-Dawley rats. Other workers (44) have confirmed the suggestion that liver protein content changed in proportion to the protein content of the diet. Anthony and Edozien (45) fed young male rats isocaloric diets containing 0.5, 1.0, 2.0, 3.0, 5.0, and 18.0% lactalbumin in ad libitum or restricted amounts. These workers reported that rats fed low protein diets showed a decrease in total liver protein in both ad libitum and restricted groups. More recent reports support the findings of earlier workers (46, 47). Coward et al. (46) reported that in ad libitum-fed rats, a decrease in dietary ratios
of protein to energy resulted in a decreased liver protein content. In restricted animals, reductions in values for protein to energy ratios had little effect on liver protein content. Quartey-Papafio et al. (47) studied young male rats fed isocaloric diets containing 20%, 8%, 4% or no protein ad libitum for a 6-day period and found a significant decrease in liver protein content with protein restriction. Thus, although the long-term effects of protein restriction have not been elucidated, it has been well established that short-term protein restriction leads to a decrease in liver protein content.

The effects of caloric restriction on liver protein content have not yet been adequately defined. Goodman and Ruderman (48) studied the effect of starvation on young male Sprague-Dawley rats and reported that hepatic protein was diminished early in the fast. However, it is known that liver protein stores are rapidly mobilized during starvation (48). Thus the effect of long-term moderate caloric restriction on liver protein could be quite different from that of starvation. Hill et al. (43) have reported an increase in liver protein in young male rats fed a diet adequate in protein but restricted in calories (60% of control). Panemangalore et al. (49) have reported no change in liver protein in a similar study. Therefore, the effect of caloric restriction on liver protein content remains to be definitively described.

D. THE EFFECTS OF AGE AND PROTEIN AND CALORIC RESTRICTION ON THE RATE OF LIVER PROTEIN SYNTHESIS

Changes in Rates of Liver Protein Synthesis with Age

It has been suggested that the natural life span of living organisms is at least to some extent under genetic control. It would
therefore be expected that alterations in protein synthesis would be age-associated (50). Thus the effects of aging on the rates of protein synthesis have been extensively studied in the tissues of laboratory animals. Many of these studies have focused on the age-associated changes in liver protein synthesis in rodents. The results of these investigations remain equivocal, possibly due to variations in age, strain, and nutritional state of the animals studied and in the methodologies utilized to measure rates of protein synthesis.

The comparison of very young with senescent rats has shown a marked decrease in protein synthetic rates with age (51). However, when mature and senescent rats have been compared, the results have become difficult to interpret. Several workers have reported that there was no change in liver protein synthesis with age in rats. No difference was reported in the rate of incorporation of $[^{14}\text{C}]$leucine into liver slices of mature (12-14 month) versus senescent (24-31 month) rats (40). Nikitin and Golubitskaya (52) found no difference in methionine incorporation into liver proteins between rats 12 and 24 months of age. Millward and Bates (53) measured the rate of liver protein synthesis by the method of continuous infusion of $[^{14}\text{C}]$tyrosine in a strain of slow growing rats at 1 and 2 years of age. These workers reported no evidence of change in the liver protein synthesis rate of these animals.

Several workers have investigated the protein synthetic capacities of liver homogenates using different types of cell-free systems. With a cell-free system, conditions for protein synthesis can be carefully controlled and the problem of measuring the specific activity of the amino acid precursor pool is eliminated (50). Cell-free protein synthesis has been shown to decrease with age in muscle, brain,
and liver of rats (54). Mainwaring (55) showed that cell-free liver protein synthesis of 30-month-old mice was 47% less than that of 5-month-old animals. Hrachovec (56) showed similar results in a study of young (7-11 month) versus old (17-22 month) animals. Liver microsomes from old rats showed an average 32% decline in levels of amino acids incorporated into protein. Buetow and Gandhi (1) studied protein synthesis measured by the incorporation of $[^{14}\text{C}]$leucine or a mixture of $[^{14}\text{C}]$ labeled amino acids into cold trichloroacetic acid-insoluble material by liver microsomes. In female Wistar rats, initial and maximal uptake levels of amino acids of liver microsome systems were lower in old (20-31 month) than in adult (12 month) rats. Thus, it was concluded that the microsomes from senescent livers were less active than those from adult livers. Other workers have recently reported similar findings using cell-free systems (54, 57, 58). Kurtz (59) reported a study of livers of 11-month- and 28-month-old mice in which an age-associated decrease in the number of active ribosomes was found. Ricca et al. (60) studied the amount of radioactively labeled valine incorporated into protein in single cell suspensions of hepatocytes. The specific activities of the intra- and extra-cellular L-valine pools were measured so that the actual rate of protein synthesis could be calculated. These workers reported that the rate of protein synthesis of hepatocytes from 1.5-month-old rats was 64% higher than was that from 18-month-old rats. A recent report (61) confirms the finding of a diminished capacity of hepatocytes from aging rats to synthesize protein.

Several reports indicate that liver protein synthesis may actually increase with age. Van Bezooijen et al. (2) investigated the protein synthesizing capacity of 3-, 12-, 24-, 31-, and 36-month-old rats by the
measurement of the incorporation of $[^{14}\text{C}]$leucine in a preparation of isolated liver parenchymal cells. The results of this work showed that the protein synthetic capacity of the liver parenchymal cells decreased between 3 and 12 months, remained constant from 12 to 24 months, and then increased between 24 and 36 months of age. Du et al. (62) reported an increase in the incorporation rate of leucine into particulate proteins in livers of old (31-32 month) mice. The most pronounced age differences were found in the microsomal fraction. These workers suggested that their findings were consistent with the earlier reports of a greater serum albumin synthesis in old animals (63, 64). A recent study of protein synthesis by intact liver parenchymal cells from male Fischer F344 rats 2.5-30 months of age measured the incorporation of $[^{3}\text{H}]$valine into acid-insoluble material (50). It was reported that the rate of protein synthesis by liver parenchymal cells decreased by 44% between 2.5 and 18 months, and then increased by 18% between 18 and 30 months of age.

The Effects of Protein and Caloric Restriction on Rates of Liver Protein Synthesis

It is known that the rate of liver protein synthesis is influenced by many nutritional factors. Although the effects of protein and caloric restriction on liver protein synthetic rates have been investigated by many workers, the results are equivocal. The discrepancies may result from several factors, including differences in nutritional state, length of treatment, and methodology used to determine rates of protein synthesis.

The effect of dietary protein on protein synthesis has been studied extensively. Dietary protein deprivation is associated with loss of
body weight and negative nitrogen balance. Since all organs do not lose protein to the same extent, different control mechanisms must be operating in various tissues. Thus, the loss of protein from a tissue takes place when the rate of protein breakdown exceeds that of synthesis. It is known that the muscles of protein-depleted rats show dramatic reductions in rates of protein synthesis and that breakdown may be increased as well (11). However, the information available regarding the effect of protein restriction on rates of liver protein synthesis is contradictory.

It has been reported that protein restriction may decrease the rate of hepatic protein synthesis. Wannemacher et al. (65) compared weanling rats fed a 6% casein diet with those fed an 18% casein diet (controls). They reported that the protein synthetic competence of isolated ribosomes from the hepatocytes of protein-restricted rats was 40-50% of that of control animals. In an investigation using a single injection of $[^{14}\text{C}]\text{Na}_2\text{CO}_3$ as a means of labeling protein, Millward (8) found a slight decrease in the rate of hepatic synthesis in rats fed a protein-free diet for 3 days. In a more recent investigation, McNurlan et al. (9) studied rates of isotope incorporation into protein after injections of large doses of $[^{14}\text{C}]\text{leucine}$. Rats fed a protein-free diet for 8 days showed a marked reduction in total protein synthesis. Sato et al. (66) have demonstrated that protein depletion causes an extensive breakdown of rat hepatic polysomes and that refeeding of an amino acid-adequate diet leads to rapid recovery of the normal polysomal profile. Likewise, Yokogoshi and co-workers (67) have reported that, in rats, polyribosomes were aggregated in proportion to the casein
content of the diet. The monomer-dimers per total ribosome reached a plateau at a dietary casein level of about 25%. In contrast, Haider and Tarver (44) reported no effect of dietary protein restriction on liver protein synthesis. These workers injected $[^{14}\text{C}]$lysine intravenously into rats fed high-(64%), normal-(27%), or low-(8%) protein, or protein-free diets. No significant differences were reported in liver protein synthetic rates regardless of dietary protein level.

Other workers have reported that protein restriction may increase the rate of hepatic protein synthesis. Waterlow and Stephen (10) measured liver protein synthesis in rats fed a 6% casein diet for as long as 10 days. These workers reported that despite rapid body weight loss the rate of protein synthesis in the liver was actually increased. Similar findings were reported by Garlick and co-workers (11) when rats were fed a protein-free diet for 21 days and the fractional rate of protein synthesis measured by continuous infusion of $[^{14}\text{C}]$tyrosine. Although the liver slowly lost protein, the fractional rate of synthesis was increased. It was suggested that protein deprivation therefore also caused an increase in the rate of liver protein breakdown. A study of the effects of amino acid and caloric restriction on protein synthesis in rats intravenously infused with $[^{15}\text{N}]$glycine was reported by Stein et al. (12). Rats fed adequate calories but no amino acids showed an increased rate of liver protein synthesis. A highly significant increase in liver protein catabolism was reported in animals fed diets deficient in calories and/or amino acids (12). In view of the conflicting reports, it must be concluded that the effect of dietary protein restriction on liver protein synthesis remains to be described definitively.
Although there is little information available regarding the effect of long-term caloric restriction on tissue protein synthesis, the effects of short periods of starvation have been documented. Waterlow and Stephen (10) studied the effect of starvation for 2 days on rats infused intravenously with $[^{14}\text{C}]$leucine. These workers found no change in liver rates of protein synthesis. This finding was supported by the report of Garlick et al. (11) who examined the fractional rates of protein synthesis in the tissues of rats continuously infused with $[^{14}\text{C}]$tyrosine and found a slight (15%) reduction in synthetic rate after 2 days of starvation.

Several workers have reported that starvation decreased rates of liver protein synthesis. Millward (8) used $[^{14}\text{C}]\text{Na}_2\text{CO}_3$ labeling of protein in order to study the effect of 3 days starvation on tissue protein synthesis of Charles River rats. He reported that starvation produced a significant decrease in the liver protein synthetic rate. Dich and Tonnesen (68) reported that isolated hepatocytes from fed rats had substantially higher rates of protein synthesis than such cells from fasted animals. McNurlan and co-workers (38) measured liver protein synthesis using massive injections of $[^{14}\text{C}]$leucine to saturate all amino acid pools to the same specific radioactivity and thus minimize the problem of measuring the specific activity of the precursor amino acid. Following starvation of rats for 2 days, they found a 30% decrease in the fractional rate of liver protein synthesis. It has also been inferred from polyribosome profiles that starvation produces a decrease in the rate of liver protein synthesis (69). Thus most current work supports the suggestion that starvation produces a decrease in hepatic
protein synthesis. The effects of long-term caloric restriction on liver synthetic rates remains to be demonstrated.
CHAPTER III
EXPERIMENTAL PROCEDURE

A. GENERAL PLAN

The male Wistar rats used in this study were obtained as weanlings from the National Research Laboratories in Creve Coeur, Missouri. When the animals were 32 days of age, they were assigned to the 6 dietary treatments shown in Table 1. Each group contained 36 animals to be sacrificed at 2 years of age (old). One year later, 12 weanling animals were assigned to each of the same 6 dietary treatments and designated to be sacrificed at 1 year of age (young).

The dietary treatments are shown in Table 1. Three groups were ad libitum-fed semisynthetic diets containing 12, 20, or 28% casein; an additional 3 groups were restricted-fed two-thirds of the feed consumed by their ad libitum-fed controls and received 18, 30, or 42% casein diets. Thus paired restricted groups consumed the same amount of protein but one-third fewer calories than their ad libitum-fed controls.

Table 2 shows the composition of the 20% casein diet. Diets varying from 20% casein were made isocaloric by substitution of equal amounts of cornstarch and sugar for casein and vice versa.

All animals were individually housed in 7" x 10" x 7" wire mesh stainless steel cages and provided water ad libitum. On a weekly basis the position of each row of cages in the racks was rotated vertically and the racks rotated within the room. Cages were checked daily and any dead animals were autopsied. The daily feed intakes of ad libitum-fed
<table>
<thead>
<tr>
<th>Protein level</th>
<th>Casein in diet</th>
<th>Ad libitum-fed</th>
<th>Restricted-fed¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Low</td>
<td>12</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>20</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>28</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

¹Restricted animals were fed two-thirds of the mean feed intake of their ad libitum-fed controls.
# TABLE 2

## COMPOSITION OF A TYPICAL DIET (20% CASEIN)

<table>
<thead>
<tr>
<th>Dietary component</th>
<th>Percent of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>20.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>29.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>29.0</td>
</tr>
<tr>
<td>Alphacel&lt;sup&gt;1&lt;/sup&gt;</td>
<td>9.0</td>
</tr>
<tr>
<td>Crisco vegetable shortening</td>
<td>6.0</td>
</tr>
<tr>
<td>Salt mix&lt;sup&gt;1,3&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>Wesson vegetable oil</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;1,4&lt;/sup&gt;</td>
<td>2.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

<sup>2</sup>Determined by the Kjeldahl method to contain 91.5% protein.

<sup>3</sup>Formulated to supply the following amounts of minerals (g/kg salt mixture): CaCO₃, 543.0; MgCO₃, 25.0; MgSO₄, 16.0; NaCl, 69.0; KCl, 112.0; KH₂PO₄, 212.0; FePO₄·4H₂O, 20.5; KI, 0.08; MnSO₄, 0.35; NaF, 1.00; Al₂(SO₄)K₂SO₄, 0.17; and CuSO₄, 0.90.

<sup>4</sup>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 B₁₂, 0.00135; and alpha-tocopherol, 5.0. Sufficient dextrose was added to make 1 kg.
rats were measured on approximately one-third of the rats on a rotating basis until the animals were 1 year of age. During the second year of life, the mean daily feed consumption of the ad libitum-fed controls was determined biweekly. Such intakes were used as a basis to calculate the amount of feed presented to the restricted-fed rats. All rats were weighed weekly until 6 months of age, biweekly until 1 year of age, and monthly thereafter.

B. \([U-^{14}C]TYROSINE FEEDING\)

Three days prior to sacrifice rats selected randomly were meal trained to eat 10 g of their respective diets during a 6-hour period each morning. Water was added to the diets to achieve a consistency similar to the diets fed on the day of sacrifice. On the day of sacrifice the animals, along with their respective agar diets, were placed in polycarbonate metabolic chambers as shown in Figure 1. Each diet preparation contained 10 g of semisynthetic diet, 8 g aqueous agar solution (3.75% agar), and 1.5 µCi L-[U-\(^{14}\text{C}]\)tyrosine per 100 g body weight (13). Expired CO\(_2\) was collected in 15 ml of an ethanolamine:ethylene glycol monomethyl ether (1:2, v/v) solution for 0.5-hour intervals for the first 3 hours and for 1-hour intervals for the next 3 hours. Radioactivity \((^{14}\text{C})\) of the CO\(_2\) trapping solution was determined by adding 3 ml of the solution to 15 ml of a scintillation fluid containing toluene:ethylene glycol monomethyl ether (2:1, v/v) and 5.5 g PPO/liter of solution. Timing was started when the rats began eating. Usually the diet was consumed during the first 2 hours of the period. At the end of the 6-hour period the animals were removed from the
Figure 1. Metabolic chamber and apparatus for collecting expired CO₂.
metabolic chamber, stunned by a blow to the head, and decapitated. Livers were removed, weighed, and stored at -60° until analyzed for protein and free and protein-bound tyrosine specific activity.

C. LIVER DETERMINATIONS

Protein Content

Liver protein was isolated by a modification of the method of Shibko et al. (70) and dissolved in NaOH. The biuret test (71) used for color development depends upon the formation of complex ions between copper and proteins in an alkaline solution, which produces a violet color that is measurable spectrophotometrically.

Reagents.

1. Perchloric acid solution (HClO₄), 0.6% (w/v): 2.6 ml of 70% HClO₄ was diluted to 500 ml with 95% ethanol.

2. Biuret reagent: 3 g CuSO₄·5H₂O, 9 g NaKC₄H₄O₆·4H₂O, 8 g NaOH and 5 g KI were dissolved, in order, and diluted to 1 liter with water and stored in a polyethylene bottle.

3. Bovine serum albumin stock standard: 1 g bovine serum albumin was dissolved in and diluted to 100 ml with 0.4 N NaOH and allowed to stand overnight. Working standards were prepared daily by diluting 0.0-0.8 ml of the stock standard to 1 ml with 0.4 N NaOH.

Procedure. One ml of 5% (w/v) liver homogenate prepared with cold distilled water (Polytron, Brinkman Instruments, Westbury, N.Y.) was pipetted into duplicate tubes. To each tube was added 0.1 ml of 70% HClO₄. The contents of each tube were mixed and chilled on ice for 10 minutes. The tubes were then centrifuged (HN-SII, Damon/IEC Division, Needham Hts., Mass.) for 10 minutes at 600 x g. The resulting
supernatant solutions were discarded and the precipitates washed with 3.0 ml of 0.6% perchloric acid. Tubes were centrifuged at 600 x g for 10 minutes. Supernatant solutions were again discarded and the precipitates washed with 3.0 ml of ether and centrifuged at 600 x g for 10 minutes. The supernatant solutions were discarded again and the protein precipitates were dissolved in 3.0 ml of 0.4 N NaOH and allowed to stand overnight. To achieve color development, 4.0 ml of biuret reagent was added to 1 ml of working standard or sample protein solution in each tube. The contents of each tube were mixed, incubated for 30 minutes in a 45° waterbath, and allowed to cool for 5 minutes. The absorbance of each sample and standard was determined using a spectrophotometer (Model 24, Beckman Instruments, Palo Alto, Ca.) at 550 nm. Liver protein content was calculated as follows:

\[
\text{Protein, mg/100 mg liver} = \frac{\text{Conc of std}}{\text{A of std}} \times \text{A of sample} \times 6
\]

**Free and Protein-Bound Tyrosine Specific Activity**

Trichloroacetic acid (TCA) was used to precipitate liver protein and diethyl ether was then used to extract TCA from the supernatant solution (39). A modification of the method of Shibko et al. (70) was used to extract nucleic acids and lipids from the protein precipitate, which was then hydrolyzed to constituent amino acids. The method of Garlick and Marshall (72) was used to convert the tyrosine in both the supernatant solution and the protein hydrolysate to tyramine using L-tyrosine decarboxylase. Tyramine was extracted from an alkaline salt solution into ethyl acetate and then recovered in dilute H₂SO₄. The aqueous solution was analyzed for tyramine using the fluorometric
nitrosonapthol method of Waalkes and Udenfriend (73) and its $^{14}$C radioactivity determined by liquid scintillation spectrometry.

Reagents.

1. Trichloroacetic acid ($\text{Cl}_3\text{CCO}_2\text{H}$), 100, 35, and 10% (w/v): to prepare the 100% solution, 500 g of TCA was diluted to 500 ml with water. The 35 and 10% solutions were prepared by diluting 35 and 10 ml, respectively, of 100% TCA to 100 ml with water.

2. Perchloric acid ($\text{HClO}_4$), 1.5% (w/v): 12.9 ml of 70% $\text{HClO}_4$ was diluted to 1 liter with water.

3. Perchloric acid solution ($\text{HClO}_4$), 0.6% (w/v): 2.6 ml of 70% $\text{HClO}_4$ was diluted to 500 ml with 95% ethanol.

4. Ethanol-chloroform, 3:1 (v/v): 300 ml 95% ethanol was mixed with 100 ml chloroform.

5. Ethanol-ether, 3:1 (v/v): 300 ml of 95% ethanol was mixed with 100 ml ether.

6. Hydrochloric acid ($\text{HCl}$), 6 N: 260 ml concentrated $\text{HCl}$ was diluted to 500 ml with water.

7. Sodium citrate buffer, 0.5 M, pH = 5.6: 52.5 g citric acid ($\text{H}_3\text{C}_6\text{H}_5\text{O}_7$) and 73.5 g sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7\cdot2\text{H}_2\text{O}$) were diluted to 1 liter with water. The pH was adjusted to 5.6 with NaOH. The solution was kept refrigerated.

8. L-tyrosine decarboxylase suspension (1 U/0.5 ml): 25 mg of tyrosine decarboxylase was washed with 5.0 ml of 0.5 M citrate buffer and centrifuged for 5 minutes at 900 x g at 0° (Model J-6B, Beckman Instruments, Palo Alto, Ca.). The pellet obtained was resuspended in 0.5 ml of 0.5 M citrate buffer and mixed. The suspension was
subsequently mixed for 15 seconds before each pipetting. The preparation was kept on ice at all times and used within 1 hour.

9. Sulfuric acid ($\text{H}_2\text{SO}_4$), 1:500 (v/v): 4 ml of concentrated $\text{H}_2\text{SO}_4$ was added to 2000 ml water.

10. Tyramine stock standard ($\text{HO(C}_6\text{H}_4\text{)}\text{CH}_2\text{CH}_2\text{NH}_2$), $10^{-3}$ M: A stock standard was prepared by diluting 137.2 mg of tyramine to 1 liter with 1:500 $\text{H}_2\text{SO}_4$. A working solution was then prepared by diluting 100 ml of stock standard to 1 liter with 1:500 $\text{H}_2\text{SO}_4$. A series of standards was prepared daily by diluting 0.0-3.0 ml of working solution to 3.0 ml with 1:500 $\text{H}_2\text{SO}_4$.

11. Nitrosonaphthol reagent: 10 ml water and 0.2 g 1-nitroso-2-naphthol were diluted to 200 ml with absolute ethanol. The solution was allowed to stand overnight, filtered, and stored at room temperature.

12. Nitric acid ($\text{HNO}_3$), 1:4 (v/v): 200 ml of concentrated $\text{HNO}_3$ was added to 800 ml water.

13. Sodium nitrite ($\text{NaNO}_2$), 5% (w/v): 5 g of $\text{NaNO}_2$ was diluted to 100 ml with water.

14. Nitric acid reagent: 0.5 ml 5% $\text{NaNO}_2$ was mixed with 50 ml of 1:4 $\text{HNO}_3$ not more than 1 hour before use.

Procedure: Five ml of a 20% (w/v) liver homogenate prepared in cold distilled water (Polytron, Brinkman Instruments, Westbury, N.Y.) was pipetted into each of duplicate tubes. To each tube was added 2.0 ml of 35% TCA and the contents were mixed and allowed to stand in an ice bath for 10 minutes. Tubes were centrifuged (Model J-6B, Beckman Instruments, Palo Alto, Ca.) for 10 minutes at 700 x g at 0°. The supernatant solutions obtained were poured into 50-ml tubes and
kept in an ice bath; the precipitates were washed 3 times with 3.0 ml of 10% TCA and the washes added to their respective supernatant solutions. Fifteen ml of ether was added to each tube containing supernatant solution plus washes. The tubes were capped and shaken vigorously and then the ether layer was removed and discarded. The ether extraction was repeated 2 times. During the last extraction, contents of the tubes were shaken and centrifuged at 700 x g at 0° for 10 minutes before the ether layers were removed. Residual ether was evaporated from the supernatant solutions for 30 minutes in a 40° water bath. Contents of tubes were mixed at 10-minute intervals to speed ether evaporation. The supernatant solutions were then evaporated under vacuum with shaking at 55° to near dryness, e.g., 0.5 ml (Rotary Evomix 3-2100, Buchler Instruments, Fort Lee, N.J.). The sample was then dissolved in 1.0 ml of 0.5 M citrate buffer.

The tubes containing the protein precipitates were drained and to each was added 10 ml of 1.5% HClO₄. The contents were mixed and the tubes incubated in a 90° water bath for 20 minutes, with mixing every 5 minutes. Tubes were then cooled to room temperature and centrifuged at 20° for 10 minutes at 800 x g and the supernatant solutions containing nucleic acids discarded. To each precipitate was added 10 ml of 0.6% perchloric acid. The contents were mixed and centrifuged at 800 x g at 20° for 10 minutes and the supernatant solutions discarded. Ten ml of ethanol-chloroform was then added to each tube and the contents were mixed and centrifuged for 10 minutes at 800 x g at 20°. The supernatant solutions were discarded and the precipitates were resuspended in 8.0 ml of ethanol-ether and incubated in a 37° water bath.
for 15 minutes. The tubes were cooled to room temperature and 4 ml of cold (7°) hexane was then added to each tube. The contents were mixed and centrifuged at 700 x g at 20° for 10 minutes and the resulting supernatant solutions were discarded. To each tube was added 10 ml of ether and then the contents were mixed and centrifuged at 800 x g for 10 minutes at 0°. The supernatant solutions were discarded and the remaining ether was evaporated from the protein pellet, to which was then added 10 ml of 6 N HCl. Contents of the tubes were mixed and the tubes were placed in a sand bath in an 110° oven for 18 hours. The hydrolysates then were evaporated just to dryness in a 40° convection oven. The residue was dissolved in 3.0 ml of 0.5 M citrate buffer.

To each buffered solution of supernatant solution or protein hydrolysate was added 0.2 and 0.34 ml of tyrosine decarboxylase suspension, respectively. The contents of tubes were mixed and the tubes were capped and incubated for 1 hour in a 37° water bath. Tube contents were gently shaken for 30 seconds at 10-minute intervals. Following incubation, 10 ml of ethyl acetate was added to each tube and the contents mixed immediately. This was followed by the addition of 2 g of a mixture of NaCl:Na₂CO₃ (1:1,w/w). Contents of tubes were mixed immediately and then centrifuged at 700 x g for 5 minutes at 20°. To the organic layers derived from the supernatant solutions was added 3.0 ml 1:500 H₂SO₄ and to those derived from the protein hydrolysates 2.5 ml of the dilute acid. Five ml of chloroform was added to each tube and the contents were mixed vigorously and allowed to stand for 10 minutes. The tubes were centrifuged for 10 minutes at 150 x g at room temperature (HN-SII, Damon/IEC Division, Needham Hts., Mass.). The
aqueous (top) layer was removed and its radioactivity \(^{14}\text{C}\) was determined by adding 1.0 ml to 15.0 ml of Aquasol II as the scintillation fluid. All radioactivity determinations were carried out in a LS-100C Liquid Scintillation Counter (Beckman Instruments, Irvine, Ca.).

Zero-3.0 ml of tyramine working standard or 1 ml (supernatant solution) or 0.1 ml (protein hydrolysate) of the aqueous layer was pipetted into tubes and diluted to 3.0 ml with 1:500 \(\text{H}_2\text{SO}_4\). Then 1.0 ml of nitrosonaphthol reagent was added to each tube and the contents were mixed. One ml of nitric acid reagent was then added to each tube and the contents immediately mixed. Tubes were incubated in a 55\(^\circ\) water bath for 30 minutes, with mixing every 10 minutes. Tube contents were then cooled to room temperature in a water bath for 10 minutes. The excess nitrosonaphthol reagent was extracted by adding 10 ml of 1,2-dichloroethane, mixing vigorously, and allowing the tubes to stand for 10 minutes. The contents of tubes were then centrifuged for 3 minutes at 150 x \(\text{g}\) at room temperature (HN-SII, Damon/IEC Division, Needham Hts., Mass.). The top layer from each tube was pipetted into a cuvette and the fluorescence measured at 570 nm after activation at 460 nm and a sensitivity of 3 x. The specific radioactivity \(^{14}\text{C}\) for free and protein-bound tyrosine was calculated as follows:

\[
\text{Specific activity (dpm/nmole)} = \frac{\text{dpm}}{\text{F of std} \times \text{F of sample}}. 
\]

D. CALCULATION OF FRACTIONAL RATE OF PROTEIN SYNTHESIS

Liver protein synthesis was determined as a fractional rate, i.e., the proportion of protein which was synthesized per unit time (9).
The synthetic rate was therefore expressed as fraction/day. The fractional rate was calculated by the method of Swick (74), as modified by Garlick et al. (75). The equation utilized (75) was as follows:

\[
\frac{SB}{SF} = \frac{\lambda f}{\lambda f - ks} \times \frac{1}{1 - \exp(-\lambda ft)} - \frac{ks}{\lambda f - ks}
\]

where:

- \(SB\) = specific activity of protein-bound tyrosine, expressed as dpm/nmole tyramine;
- \(SF\) = specific activity of free tyrosine, expressed as dpm/nmole tyramine;
- \(t\) = time of infusion, expressed in days;
- \(\lambda f\) = rate constant for the time course of rise of specific activity of SF, expressed per day; and
- \(ks\) = rate of protein synthesis, expressed in terms of fraction per day.

Using the rate of rise \(k\) of \(^{14}\)C radioactivity in the expired \(CO_2\), the liver \(\lambda f\) was approximated \((\lambda f = k)\) for each individual rat (13). The rate constant \(k\) was calculated for each rat by fitting the \(^{14}\)CO\(_2\) production curve to the equation (34):

\[
C = C_{\text{max}} - B \exp(-kt)
\]

where:

- \(C\) = dpm at any given time;
- \(C_{\text{max}}\) = dpm at plateau;
- \(B\) = integration constant;
k = rate constant, expressed as days$^{-1}$; and
t = time, expressed in days.
The approximation of $\lambda f$ is considered adequate because it has been shown that the calculated value of $k_s$ is relatively insensitive to the value of $\lambda f$ (10). Because the equation of Garlick et al. (75) cannot be rearranged in order to solve for $k_s$, the equation was solved indirectly by a process of successive approximations, using the method of Newton's approximation available in SAS 1979 (34, 76).

E. STATISTICAL METHODS

The procedures available in GLM (general linear models) of SAS 1979 were utilized to evaluate the effects of protein and caloric restriction and age on liver protein and fractional rate of protein synthesis (76). Differences among dietary treatments and age were tested using analysis of variance (77). Specific differences among group means for the effects of calories (A vs R), protein (linear and/or quadratic) and the interaction of calories and protein (linear and/or quadratic interaction) were tested with orthogonal contrasts (78). A probability level of less than 0.05 was considered statistically significant. The least squares option in GLM was utilized to derive group means and standard errors. The FUNCAT (functional analysis of categorical variables), an option available in SAS 1979 (76), was used to test the influence of diet on percent survival at 2 years of age.
CHAPTER IV

RESULTS

Growth curves for the ad libitum- (A) and restricted-fed (R) animals are presented in Figure 2. The curves for R groups were of similar shape but at a lower level than those for A groups. Although the growth curve of rats fed the low-protein diet ad libitum was lower than those for other ad libitum groups, by 1 year of age the body weights for all A groups were similar. From age 2 to 12 months, mean feed intakes of A groups ranged from 17.4 to 19.7 g/day (R groups were fed two-thirds of the amount consumed by their ad libitum-fed controls). After 2 months of age, there was no significant difference in the feed intakes of A groups. The above data are presented as general background information and have been presented and discussed in detail by Davis. ²

The percent survival for all dietary groups is shown in Figure 3 and in Table 3. Percent survival ranged from 19.4 to 33.3% in A groups and from 30.6 to 61.1% in R groups at 2 years of age. Thus caloric restriction significantly increased (p < 0.003) the percent survival. In both caloric groups low dietary protein intake was associated with low percent survival.

Figure 4 and Table 4 present the effects of age and protein and caloric restriction on liver protein content. The number (n) of animals

Figure 2. Effect of level of dietary protein on calculated growth curves of ad libitum- (A) and restricted-fed (R) rats.
Figure 3. Effect of level of dietary protein on percent survival at 2 years of age of ad libitum- (A) and restricted-fed (R) rats.
### TABLE 3

**EFFECT OF LEVEL OF DIETARY PROTEIN ON PERCENT SURVIVAL AT 2 YEARS OF AGE OF AD LIBITUM- AND RESTRICTED-FED RATS**

<table>
<thead>
<tr>
<th>Protein level</th>
<th>Percent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad libitum</strong></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>19.4</td>
</tr>
<tr>
<td>Medium</td>
<td>30.6</td>
</tr>
<tr>
<td>High</td>
<td>33.3</td>
</tr>
<tr>
<td><strong>Restricted</strong></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>30.6</td>
</tr>
<tr>
<td>Medium</td>
<td>61.1</td>
</tr>
<tr>
<td>High</td>
<td>52.8</td>
</tr>
</tbody>
</table>

**Contrast Statements - P Values**

- Ad libitum vs restricted: \(<0.003\)
- Protein intake:
  - Linear: \(<0.03\)
  - Quadratic: NS\(^3\)
- Protein intake x caloric level:
  - Linear: NS
  - Quadratic: NS

---

1. Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

2. Ad libitum- and restricted-fed groups with same protein intake were combined.

3. \(p > 0.05\) designated as NS (nonsignificant).
Figure 4. Effect of dietary protein and age (1- versus 2-years of age) on liver protein content of ad libitum- (A) and restricted-fed (R) rats. (Data points are means ± SEM.)
TABLE 4
EFFECT OF LEVEL OF DIETARY PROTEIN AND AGE ON LIVER PROTEIN CONTENT OF AD LIBITUM- AND RESTRICTED-FED RATS

<table>
<thead>
<tr>
<th>Protein level</th>
<th>Protein (mg/100 mg)</th>
<th>Young</th>
<th>Old</th>
<th>Both ages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>17.58 (8)¹</td>
<td>17.89 (5)</td>
<td>17.73 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>17.91 (9)</td>
<td>17.70 (5)</td>
<td>17.81 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>18.72 (8)</td>
<td>17.82 (7)</td>
<td>18.27 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Restricted²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>19.05 (9)</td>
<td>19.20 (5)</td>
<td>19.13 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>19.61 (10)</td>
<td>18.42 (8)</td>
<td>19.01 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>19.97 (11)</td>
<td>19.76 (7)</td>
<td>19.87 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM for column</td>
<td>18.78 ± 0.18</td>
<td>18.46 ± 0.22³</td>
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</tr>
</tbody>
</table>

Contrast Statements - P Values

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum vs restricted</th>
<th>Protein intake⁴</th>
<th>Protein intake x calorie level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Protein intake</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Linear</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Quadratic</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Number of animals per group.
²Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.
³Values of old animals were significantly less (p < 0.05) than those of young animals.
⁴Ad libitum- and restricted-fed groups with same protein intake were combined.
⁵p > 0.05 designated as NS (nonsignificant).
per group was determined by the number of animals living at the time of sacrifice. Liver protein content was lower \((p < 0.05)\) in old than in young animals. Mean liver protein content ranged from 17.58 to 19.97 mg/100 mg in young animals and from 17.70 to 19.76 mg/100 mg in old groups. In 1-year-old animals protein restriction produced a linear decrease \((p < 0.03)\) in liver protein content. Caloric restriction produced a dramatic increase in liver protein concentration in both young \((p < 0.0001)\) and old \((p < 0.005)\) animals. Liver protein content of 1-year-old groups ranged from 17.58 to 18.72 mg/100 mg in A groups and from 19.05 to 19.97 mg/100 mg in R groups. The comparable values for old rats ranged from 17.70 to 17.89 in A groups and from 18.42 to 19.76 mg/100 mg in R groups.

Figure 5 shows a \(^{14}\text{CO}_2\) production curve generated from data obtained during a 6-hour infusion period for a 1-year-old rat ad libitum-fed a high-protein (28% casein) diet. Such a curve is an example of those obtained for the experimental animals. Mean rate constants \((\lambda_f)\) calculated from \(^{14}\text{CO}_2\) production curves ranged from 19.43 to 91.47 days\(^{-1}\) (see Appendix, Table A-1). The mean ratios of the specific activity of protein-bound tyrosine to the specific activity of free tyrosine \((\text{SB/ SF})\) are also shown in the Appendix (Table A-2). Mean values for the ratio ranged from 0.31 to 0.47.

The effects of age and diet on liver protein synthesis \((k_s)\) are shown graphically in Figure 6 and mean values for \(k_s\) (fraction/day) and results of statistical analysis of the data are presented in Table 5. With age, there was a significant \((p = 0.02)\) increase (14%) in the fractional rate of liver protein synthesis. Mean \(k_s\) values ranged from 1.99 to 2.73 fraction/day in young animals and from 1.84 to 3.32 fraction/day in old groups. Protein restriction produced a linear
Figure 5. Typical $^{14}$CO$_2$ production curve generated by fitting the equation $C = C_{\text{max}} - B \exp(-kt)$ to experimental data obtained during a 6-hour infusion period. (Data shown were obtained for a young rat, 1-year, ad libitum fed a high-protein diet. $C_{\text{max}} = 6.8 \times 10^4$ dpm; $B = -9.6 \times 10^4$; $k = 21.11$ days$^{-1}$.)
Figure 6. Effects of dietary protein and age (1- versus 2-years of age) on rates of liver protein synthesis of ad libitum- (A) and restricted-fed (R) rats. (Data points are means ± SEM.)
TABLE 5
EFFECT OF LEVEL OF DIETARY PROTEIN AND AGE ON MEAN FRACTIONAL RATES OF LIVER PROTEIN SYNTHESIS OF AD LIBITUM- AND RESTRICTED-FED RATS

<table>
<thead>
<tr>
<th>Protein level</th>
<th>Synthetic rate (fraction/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
</tr>
<tr>
<td>Ad libitum</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2.46 (8)</td>
</tr>
<tr>
<td>Medium</td>
<td>2.62 (9)</td>
</tr>
<tr>
<td>High</td>
<td>2.46 (8)</td>
</tr>
<tr>
<td>Restricted²</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>2.73 (9)</td>
</tr>
<tr>
<td>Medium</td>
<td>2.01 (10)</td>
</tr>
<tr>
<td>High</td>
<td>1.99 (11)</td>
</tr>
<tr>
<td>Mean ± SEM for column</td>
<td>2.38 ± 0.08</td>
</tr>
</tbody>
</table>

Contrast Statements - P Values

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum vs restricted</th>
<th>Protein intake⁵</th>
<th>Protein intake x caloric level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS⁴</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Number of animals per group.
² Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.
³ Values of old animals were significantly greater (p = 0.02) than those of young animals.
⁴ p > 0.05 designated as NS (nonsignificant).
⁵ Ad libitum- and restricted-fed groups with same protein intake were combined.
increase ($p = 0.01$) in the protein synthetic rate of ad libitum- and restricted-fed old animals. The same tendency was noted in young restricted animals ($p = 0.06$). In 2-year-old animals caloric restriction resulted in a dramatic decrease ($p < 0.0001$) in the liver $ks$ value. In A groups the mean $ks$ for old groups ranged from 3.01 to 3.32 fraction/day while such values for R groups ranged from 1.84 to 2.90. A similar tendency was observed in young groups ($p < 0.10$). In these animals $ks$ values ranged from 2.46 to 2.62 in A groups and from 1.99 to 2.73 fraction/day in R groups. A significant interaction ($p < 0.02$) was observed between protein intake and caloric level, that is, R groups showed a linear increase in $ks$ with protein restriction while A groups showed little change (Figure 6).
CHAPTER V

DISCUSSION

The results of the present investigation indicate a significant decrease in liver protein content with age and are not in agreement with previous reports (40-42). Studies by Barrows et al. (41, 42) have shown no difference in liver protein nitrogen in young (12-14 month) versus old (24-27 month) McCollum rats of both sexes. Beauchene et al. (40) reported liver protein content values ranging from 16.2 to 17.8 mg/100 mg in young (10-14 month) and from 16.5 to 17.0 mg/100 mg in senescent (24-31 month) rats and thus found little change in liver protein content with age. Liver protein values obtained in the present study were somewhat higher than those reported by Beauchene et al. (40) but similar to those reported by others (44, 45).

In 1-year-old animals, dietary protein restriction produced a linear decline in liver protein content consistent with that previously described (43). Hill et al. (43) reported that protein restriction, with and without caloric restriction, produced a decrease in the liver protein content of weanling Sprague-Dawley rats. The present results are also supported by those of Anthony and Edozien (45) in a study of young male rats fed low-protein diets in ad libitum or restricted amounts. A decrease in liver protein content in response to dietary protein restriction has been reported by others (44, 46, 47) and is confirmed by the present investigation. The absence of a significant effect of dietary protein intake on the liver protein content of old animals does not agree with the reports of others (43-47). The dramatic
increase in liver protein content produced by caloric restriction agrees with the findings of Hill et al. (43), who evaluated the effect of caloric restriction without simultaneous protein restriction. In rats fed a high protein diet in restricted amounts (60% of control), liver protein content was 23.0 as compared to 19.8 mg/100 mg in ad libitum-fed animals (43). Panemangalore et al. (49) reported no effect of caloric restriction on liver protein content of weanling rats; however, these workers employed a more severe energy restriction than that used in the present study.

The $^{14}\text{CO}_2$ production curves generated in the present investigation were similar in shape to those reported by Harney et al. (13) and the calculated rate constants ($\lambda_f$) were also within the range reported by those workers. The method utilized in the present study to calculate the rate constant allowed the use of a computer program to fit an asymptotic function directly to the experimental data ($^{14}\text{CO}_2$) and thus avoided estimating $C_{max}$ for each rat subjectively. In addition, the function utilized does not assume that the curve generated must pass through the origin. Therefore, the method utilized for rate constant calculation was felt to be superior to that used previously by Harney et al. (13). The ratio of the specific activity of protein-bound tyrosine to the specific activity of free tyrosine was higher than that reported by Garlick et al. (11). However, Garlick et al. (11) utilized continuous intravenous infusion rather than dietary infusion of $[^{14}\text{C}]$tyrosine. The ratio was not reported in the dietary infusion paper of Harney et al. (13).

The liver protein synthetic rate observed was greater in old than in young animals and this finding is consistent with the reports
of others (62-64). Coniglio et al. (50) observed an 18% increase in the incorporation of $[\text{3H}]$valine into liver acid-insoluble material in rats 30 months of age as compared with rats 18 months of age. This increase is similar to the 14% increase observed between 12 and 24 months in the present study. Van Bezooijen et al. (2) reported an age-associated increase in protein synthetic capacity of liver parenchymal cells that occurred only after 24 months of age. The present findings are inconsistent with those from studies of cell-free protein synthesis (1, 54-59), which have shown a decrease in liver protein synthesis with age. However, because the rate of liver protein synthesis in cell-free systems is only 1% of the rate observed in vivo (60), the physiological significance of these findings remains to be defined. Present findings also conflict with reports of no change in liver protein synthesis with age (40, 52). Millward and Bates (53) showed no change in the liver protein fractional rate of synthesis with age (1 versus 2 year rats). However, these investigators used the method of continuous intravenous infusion of $[^{14}\text{C}]$tyrosine and the animals used in their study were of a slow-growing strain and therefore might not show a difference in synthetic rates between 1 and 2 years of age.

Results of the present study showed that protein restriction was associated with an increase in the fractional rate of liver protein synthesis. These findings are in agreement with those of others using the method of continuous intravenous infusion (10, 11). In rats fed a 6% casein diet ad libitum, Waterlow and Stephen (10) found an increase in the rate of liver protein synthesis despite a rapid loss in body weight. Garlick and co-workers (11) reported similar findings in rats
fed a protein-free diet for 21 days. In addition to confirming the work of Waterlow and Stephen (10) and Garlick et al. (11), Stein et al. (12) showed that liver protein catabolism was increased in animals fed diets deficient in calories and/or amino acids. Haider and Tarver (44) reported no effect of dietary protein restriction on protein synthetic rates of the liver. However, these workers utilized the "pulse dose" method to administer $^{14}$Clysine and this procedure fails to account for the specific activity of the labeled precursor amino acid. The results of the present investigation conflict with reports of diminished liver protein synthetic capacity associated with protein deprivation, as assessed by a number of other techniques (8, 65). McNurlan et al. (9) showed a marked reduction in liver protein synthesis in rats fed a protein-free diet for 8 days and injected with massive doses of $^{14}$C leucine. Although this technique allows the measurement of total liver protein synthesis rather than that of non-secreted hepatic proteins only, as does the method of continuous infusion, McNurlan et al. (9) do not feel that this difference explains the conflicting results obtained. The present findings also conflict with some reports of polysome breakdown—which is associated with reduced rates of protein synthesis—in response to protein depletion (66, 67).

The observation that caloric restriction dramatically decreased the liver protein synthetic rate is in agreement with previous studies using a variety of techniques to determine the synthetic rate (68). Millward (8) used a single injection of $^{14}$C$\text{Na}_2\text{CO}_3$ to label protein of Charles River rats starved for 3 days and showed a significant reduction in the liver protein synthetic rate. McNurlan and co-workers (38) demonstrated that 2 days of starvation produced a 30% decrease in
the fractional rate of liver protein synthesis of rats injected with massive doses of $[^{14}\text{C}]$leucine. The present results are also supported by the results of polysome profiles, i.e., starvation produces a decline in liver protein synthetic capacity (69). Using the method of continuous infusion of $[^{14}\text{C}]$leucine, Waterlow and Stephen (10) showed no effect of starvation for 2 days on the liver protein synthetic rate of rats. However, Garlick et al. (11) later demonstrated a slight reduction in liver protein synthesis with 2 days of starvation using rats continuously infused with $[^{14}\text{C}]$tyrosine. The present findings are in agreement with studies of the effect of short periods of caloric deprivation on liver protein synthesis and further describe the effects of long-term caloric restriction on synthetic rates.

Caloric restriction significantly increased the percent survival of the animals in the present investigation and this finding is in agreement with previous findings from this laboratory as well as those of others (14-24). While caloric restriction produced a greater effect on survival, dietary protein level also produced a significant effect. The association of low dietary protein intake with low percent survival in the present study is supported by the findings of Ross and Bras (33, 79), who showed a positive correlation between the amount of protein consumed and life expectancy for rats fed diets ad libitum or in restricted amounts. The present results are not in agreement with

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other reports, e.g., that dietary protein restriction has no effect on life span (28) or prolongs it (27).

Increased liver protein synthesis with age in rats has been associated with increased urinary albumin excretion during aging (2, 40). The relationship of the present findings to this observation is unclear since the method utilized in the present investigation measured the synthetic rate of fixed protein (38) rather than that of secreted protein, e.g., albumin.

In the present investigation caloric restriction produced a dramatic reduction in liver protein synthesis and increased percent survival of the animals. Protein restriction was associated with an increase in the liver protein synthetic rate and a reduction in percent survival. With age, a significant increase in protein synthesis was observed. Caloric restriction delayed the age-associated increase in liver protein synthesis. Thus, these findings support the hypothesis of Barrows (80) that the mechanism by which caloric restriction increases longevity is through decreased use of the genetic code for protein synthesis that reduces genetic imperfections that may occur during aging.

Dietary restriction has been shown to delay the onset of disease but the mechanism by which this effect is achieved has not been defined (17). The life span of an organism may be determined by the onset and severity of disease and/or by changes at the level of the genome. That is, the increase in percent survival observed in the present study in response to caloric restriction or increased protein intake may be directly related to the decrease in liver protein synthesis observed,
and the subsequent reduction in damage to the genetic code, or may be related to mechanisms that were not investigated in the present study.
CHAPTER VI

SUMMARY

The effects of protein and caloric restriction and age on liver protein content and the protein synthetic rate of male rats were investigated. The method of continuous dietary infusion of $[^{14}\text{C}]$tyrosine was utilized to determine the fractional rate of liver protein synthesis.

Growth curves for restricted-fed (R) rats were of similar shape but at a lower level than those for ad libitum-fed (A) groups. Measurement of mean daily feed intake showed little difference in A groups after 2 months of age (R groups were fed two-thirds of the amount consumed by their ad libitum-fed controls).

Caloric restriction produced a significant increase ($p < 0.003$) in the percent survival of R groups. Although caloric restriction resulted in a greater effect on life span, an effect of dietary protein intake was also shown. Low dietary protein intake was associated with a low percent survival in both caloric groups.

A decrease ($p < 0.05$) in liver protein content was observed with age. In 1-year-old animals only, protein restriction resulted in a linear decrease ($p < 0.03$) in liver protein content. In both young (1-year) and old (2-year) animals caloric restriction produced a dramatic increase ($p < 0.0001$) in liver protein concentration.

The $^{14}\text{CO}_2$ production curves generated from data obtained during a 6-hour infusion period were used to calculate the rate constant for each rat. The rate constant and the specific radioactivity of the free and protein-bound liver tyrosine were then used to calculate the
fractional rate of protein synthesis. The liver protein synthetic rate showed a significant (p = 0.02) increase (14%) with age. Dietary protein restriction produced a linear increase (p < 0.01) in the rate of liver protein synthesis of ad libitum- and restricted-fed old animals. The same tendency was observed in young restricted-fed animals. Caloric restriction resulted in a dramatic decrease (p < 0.0001) in the liver protein synthetic rate in 2-year-old animals. Young restricted-fed groups showed a similar tendency. Rates of protein synthesis exhibited a significant interaction (p < 0.02) between protein intake and caloric level, that is, R groups showed a linear increase in protein synthetic rate with protein restriction while A groups showed little change.

Caloric restriction delayed the age-associated increase in liver protein synthesis and increased percent survival, while protein restriction was associated with an increase in the liver protein synthetic rate and a reduction in percent survival. These findings support the hypothesis (80) that the mechanism by which caloric restriction increases longevity is through decreased use of the genetic code for protein synthesis, which reduces genetic imperfections that may occur during aging.
LITERATURE CITED
LITERATURE CITED


APPENDIX
### TABLE A-1

**EFFECT OF LEVEL OF DIETARY PROTEIN AND AGE ON RATE CONSTANTS \((\lambda f)^1\) OF AD LIBITUM- AND RESTRICTED-FED RATS**

<table>
<thead>
<tr>
<th>Protein level</th>
<th>Rate constant ((\text{days}^{-1}))</th>
<th>Young</th>
<th>Old</th>
<th>Both ages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ad libitum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>32.15</td>
<td>19.43</td>
<td></td>
<td>25.79 ± 12.07</td>
</tr>
<tr>
<td>Medium</td>
<td>24.28</td>
<td>39.01</td>
<td></td>
<td>31.64 ± 11.81</td>
</tr>
<tr>
<td>High</td>
<td>37.59</td>
<td>27.66</td>
<td></td>
<td>32.62 ± 10.96</td>
</tr>
<tr>
<td><strong>Restricted</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>53.65</td>
<td>78.10</td>
<td></td>
<td>65.88 ± 11.81</td>
</tr>
<tr>
<td>Medium</td>
<td>76.73</td>
<td>91.47</td>
<td></td>
<td>84.10 ± 10.04</td>
</tr>
<tr>
<td>High</td>
<td>30.97</td>
<td>35.34</td>
<td></td>
<td>33.16 ± 10.24</td>
</tr>
</tbody>
</table>

Mean ± SEM for column 42.56 ± 5.75 48.50 ± 7.10³

**Contrast Statements - P Values**

- **Ad libitum vs restricted**
  - Protein intake
    - Linear: NS
    - Quadratic: NS
  - Protein intake x caloric level
    - Linear: NS
    - Quadratic: NS

1. Using the rate of rise \((k)\) of \(^{14}\text{C}\) radioactivity in the expired \(\text{CO}_2\), \(\lambda f\) was approximated \((\lambda f = k)\). See text, p. 28.

2. Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

3. Rate constants \((\lambda f)\) were not significantly different between young and old animal groups.

4. \(p > 0.05\) designated as NS (nonsignificant).

5. Ad libitum- and restricted-fed groups with the same protein intake were combined.
TABLE A-2
EFFECT OF LEVEL OF DIETARY PROTEIN AND AGE ON SB/SF\(^1\) OF AD LIBITUM- AND RESTRICTED-FED RATS

<table>
<thead>
<tr>
<th>Protein level</th>
<th>Young</th>
<th>Old</th>
<th>Both ages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad libitum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.41</td>
<td>0.47</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Medium</td>
<td>0.40</td>
<td>0.45</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>High</td>
<td>0.40</td>
<td>0.44</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Restricted(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.44</td>
<td>0.46</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>Medium</td>
<td>0.36</td>
<td>0.38</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>High</td>
<td>0.33</td>
<td>0.31</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Mean ± SEM for column 0.39 ± 0.01 0.42 ± 0.01\(^3\)

Contrast Statements - P Values

<table>
<thead>
<tr>
<th>Statement</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Ad libitum vs restricted</td>
<td>NS(^4) &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>Protein intake(^5)</td>
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</tr>
<tr>
<td>Linear</td>
<td>&lt;0.02   &lt;0.002 &lt;0.0001</td>
</tr>
<tr>
<td>Quadratic</td>
<td>NS      NS       NS</td>
</tr>
<tr>
<td>Protein intake x caloric level</td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>&lt;0.04   NS       &lt;0.005</td>
</tr>
<tr>
<td>Quadratic</td>
<td>NS      NS       NS</td>
</tr>
</tbody>
</table>

\(^1\) Ratio of specific activity of protein-bound tyrosine to specific activity of free tyrosine.

\(^2\) Restricted-fed rats were fed one-third less calories but the same amount of protein as their ad libitum-fed controls.

\(^3\) SB/SF was not significantly different between young and old animal groups.

\(^4\) \(p > 0.05\) designated as NS (nonsignificant).

\(^5\) Ad libitum- and restricted-fed groups with the same protein intake were combined.
VITA

Connie Watkins Bales was born in Morristown, Tennessee, on October 21, 1954. She attended Talbott Elementary School and graduated from Jefferson High School in Jefferson City, Tennessee, in June of 1972. The following September she entered The University of Tennessee, Knoxville, Tennessee. In 1975 she married Jerad Bales and the following year was graduated with a Bachelor of Science in Home Economics with highest honors. In August of 1976 she accepted the position of Therapeutic Dietitian at St. Mary's Medical Center in Knoxville, Tennessee. Her work toward the Doctor of Philosophy degree in Home Economics was begun in September of 1977. During her graduate work, she held an appointment as graduate research assistant for the Agricultural Experiment Station in the Department of Nutrition and Food Sciences. After receipt of the Doctor of Philosophy degree with a major in Home Economics in August of 1981, she accepted the position of Assistant Professor in the Division of Nutrition and Foods in the Department of Home Economics at the University of Texas at Austin, Texas.