Metabolism of Collagen and Muscle Protein of Adult Rats in Protein Depletion and Repletion

Hideyuki Tanaka, Masami Nakajima, Yasuhiro Morishita, Kazuhiro Terauchi and Hiroshi Nishi

Department of Agricultural Chemistry, Faculty of Agriculture, Utsunomiya University, Mine-350, Utsunomiya 321, Japan

Received January 30, 1981

The effect of protein depletion and repletion on the contents of rat body collagen and muscle protein has been investigated. Adult male rats were placed on a protein-free diet for 64 days, and thereafter fed with a 20% casein diet for 83 days. Total nitrogen, hydroxyproline and N\(^{\alpha}\)-methylhistidine were determined in several tissues. Skin collagen was fractionated into neutral salt-soluble, acid-soluble and insoluble collagen.

In prolonged protein depletion, the total amounts of liver protein, skin collagen and muscle protein were markedly decreased, while carcass collagen (excluding skin) showed little effect. The concentrations of skin and carcass collagen were significantly increased in protein depleted rats. Some differences in metabolism between skin collagen and other carcass collagen were discussed.

After 64 days of depletion, refeeding on an adequate protein diet brought about a rapid increase in body weight, liver protein and muscle protein. Skin collagen did not increase in the early period of refeeding, but increased rapidly at a later stage. A remarkable change in the solubility pattern of skin collagen was observed in protein depletion and repletion.

These results indicate that feeding depleted rats with an adequate protein diet brings about an almost complete recovery of muscle protein and collagen which are the main sources of the amino acid pool for protein synthesis during prolonged protein deprivation.

It has been known that collagen which accounts for about one-third of the total body protein in mammals is metabolically relatively inert compared to other body proteins.\(^{1,2}\) Picou et al.\(^{3}\) found that in malnourished children, body collagen continued to increase in amount with age owing to the growth of the skin and skeletal system in spite of severe protein deficiency. On the other hand, a significant loss of collagen, particularly from the skin, in protein depleted animals has been demonstrated by several workers.\(^{4-7}\)

In the previous paper,\(^{8}\) we reported that carcass (including the skin) collagen in the adult rats was significantly lost during prolonged protein depletion and that in the early stage of protein depletion, liver and blood proteins were utilized as the sources of amino acid pools for protein synthesis, but at a later stage muscle protein and collagen were mainly utilized. Allison and Wannemacher\(^{9}\) suggested that the carcass (mostly muscles) and skin are the major sources of the over-all protein reserves in the body. Moreover, Allison et al.\(^{10}\) defined protein reserves as those tissue proteins that can be reversibly depleted and repleted, thereby contributing to the free amino acid pools of the body during depleting processes.

In the present study, we examined metabolic responses of body proteins, particularly collagen and muscle protein, to protein depletion and repletion in adult rats. Since many workers\(^{4-6,12}\) so far have shown that the turnover rate of skin collagen is different from that of other tissue collagen such as bone or tendon, in the present study, skin was removed from the other carcass and analyzed separately. Furthermore, it has been shown by some workers\(^{11-15}\) that metabolic activities of several fractions of collagen with different solubility to the various solvents are different from
Animals and diets. Forty adult male rats of the Wistar strain, weighing about 430 g, were preliminarily fed a 20% casein diet for 7 days. The animals were then divided into eight groups of 5 rats each. At the start of experiment the rats in one group were killed to serve as the initial controls for tissue analyses. Two groups were fed with the 20% casein diet throughout the experimental period as the age controls. The other 5 groups (experimental groups) were fed with a protein-free diet for 64 days and thereafter they were transferred to the 20% casein diet for 83 days. After periods of 64, 72, 80, 100 and 147 days, the rats in one experimental group were killed for tissue analyses, and after 64 and 147 days the rats in one age control group.

The composition of the experimental diets is shown in Table I. The animals were housed in individual wire cages with free access to food and water, and maintained in a temperature-controlled room (about 24°C) with a 12 hr light-12 hr dark cycle. Body weight was recorded on 8 to 10 days intervals during the experimental period.

Analysis of total nitrogen, hydroxyproline and \( N^\prime \)-methylhistidine. Each animal was killed by decapitation and exsanguinated. The fur was shaved off from the whole body except head, tail and the lower parts of the legs by electric clippers for small animals and a part of the skin from the ventral region (about 1 g) was immediately taken for the fractionation of skin collagen. The remaining shaved fur (skin) was removed, weighed and lyophilized. The lyophilized skin was defatted by extraction with diethyl ether and cut up into small pieces with scissors. After removal of the skin, the liver was removed, lyophilized and stored for later analyses. The skinned carcass combined with the collected blood was weighed and stored in a deep-freezer maintained at -20°C. The frozen carcass was cut into small pieces with a chopper and then minced several times with a meat grinder until the sample appeared to be homogeneous.

Total nitrogen in liver, skin and carcass was determined by the Kjeldahl method. After the extraction with hot 5% trichloroacetic acid, an aliquot of skin and carcass extracts was hydrolyzed in 6N hydrochloric acid at 110°C for 24 hr in a sealed tube. To remove hydrochloric acid, hydrolysate was evaporated to dryness in vacuo and this procedure was repeated several times. Hydroxyproline in skin and carcass hydrolysates was determined by the method of Firschein and Shill. \( N^\prime \)-Methylhistidine in carcass hydrolysate was determined by the method of Nishizawa et al. using a JEOL-5AH amino acid analyzer.

Fractionation of skin collagen. Various solvent systems for the fractionation of skin collagen into soluble and insoluble forms have been used by many workers. In this experiment, rat skin collagen was fractionated by extraction with 0.45M sodium chloride and 0.5M acetic acid according to the method of Heikkinen and Kulonen with a slight modification. Skin from the ventral region was placed on an ice-cold plastic plate in a cooled room with dry ice, and the surface was scraped free of any adhering hair, fat or subcutaneous tissue and finely chopped up with a scalpel. Minced skin (1 g) was extracted with 5 ml of 0.45M NaCl at 4°C with constant stirring, using a magnetic stirrer for 48 hr. The extract was centrifuged at 30,000 \( \times g \) for 2 hr, and the supernatant solution was dialyzed against running water overnight. Retentate was referred to as neutral salt-soluble collagen. The residue after the extraction with 0.45M NaCl was extracted with 0.5M acetic acid at 4°C for 24 hr with constant stirring, and the extract was then centrifuged at 30,000 \( \times g \) for 45 min. The supernatant solution was referred to as acid-soluble collagen, and the residue as insoluble collagen. These three collagen fractions of the skin were hydrolyzed in 6N HCl at 110°C for 24 hr, and hydroxyproline contents were determined as described above.

Statistical analysis. The results were tested by analysis of variance, and the Least Significant Difference test was used to evaluate the statistical significance with probability levels, 0.05 and 0.01.

RESULTS

Changes in body weight and nitrogen in tissues

Body weight changes of control and experimental groups are shown in Fig. 1. In the experimental groups, the body weight of rats fed with a protein-free diet for 64 days decreased linearly, and that on 64 days was 59%
Collagen and Muscle Protein in Protein Depleted and Repleted Rats

![Graph showing body weight changes](image)

**FIG. 1.** Body Weight Changes for Adult Rats Fed with 20% Casein Diet (Age Control) and Protein-Free Diet Followed by Protein Refeeding (Experimental).

Each point represents the mean weight of five rats in each group.

O--O, age control group; ●—●, experimental group.

...of the initial and 49% of the age control, respectively. Refeeding, after 64 days of depletion, with a 20% casein diet brought about a rapid recovery and the body weight returned to the level of initial control for about 36 days after refeeding (on 100 days after the start of experiment). In the age control groups, on the other hand, the body weight increased slowly throughout the experimental period of 147 days. Hence, the body weight of the experimental group could not reach the level of the age control group even on the last day of the experiment (on day 147).

The total nitrogen and its concentration in the carcass, skin and liver in each group are shown in Tables II and III. After 64 days of feeding with the protein-free diet, the total amount of nitrogen in each tissue was markedly decreased and that in the carcass, skin and liver was 58, 56 and 33% of the age control values, respectively. When the rats were refed with the adequate protein diet, these total nitrogen were rapidly increased.

No remarkable change in total nitrogen concentration (per unit tissue weight) was observed in the carcass and skin. But in the liver the value was significantly decreased after 64 days of depletion, and rapidly returned to normal by refeeding with the adequate protein diet. So, the decrease in the total amount of nitrogen in the carcass and skin in protein depletion appears to be attributable to the decrease of these tissue weights.

**Changes in hydroxyproline and N\(^1\)-methylhistidine in tissues**

Total amounts of hydroxyproline of the carcass and skin and of N\(^1\)-methylhistidine of the carcass in each group are shown in Fig. 2. The concentrations of hydroxyproline and N\(^1\)-

**Table II. Total Nitrogen of Carcass, Liver and Skin in Both Control and Experimental Groups**

The values are mean±standard errors of five rats. Rats in the experimental group were fed with the protein-free diet for 64 days and thereafter they were fed with the adequate protein diet for 83 days. The Least Significant Difference (L.S.D.) test is taken as the criterion of statistical significance with probability levels, 0.05 and 0.01.

<table>
<thead>
<tr>
<th>Days on diet</th>
<th>Carcass nitrogen (g)</th>
<th>Liver nitrogen (mg)</th>
<th>Skin nitrogen (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>10.2±0.4</td>
<td>—</td>
<td>411±35</td>
</tr>
<tr>
<td>64</td>
<td>11.2±0.3</td>
<td>6.5±0.3</td>
<td>485±32</td>
</tr>
<tr>
<td>72</td>
<td>—</td>
<td>8.2±0.4</td>
<td>—</td>
</tr>
<tr>
<td>80</td>
<td>—</td>
<td>8.8±0.3</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>10.1±0.3</td>
<td>—</td>
</tr>
<tr>
<td>147</td>
<td>13.0±0.9</td>
<td>11.4±0.4</td>
<td>521±23</td>
</tr>
</tbody>
</table>

L.S.D.\(_{0.05}\) 1.35 74.7 0.61
L.S.D.\(_{0.01}\) 1.80 100.2 0.82
Table III. Nitrogen Concentration of Carcass, Liver and Skin in Both Control and Experimental Groups

The values are mean ± standard errors of five rats. The Least Significant Difference (L.S.D.) test is taken as the criterion of statistical significance with probability levels, 0.05 and 0.01.

<table>
<thead>
<tr>
<th>Days on diet</th>
<th>Carcass nitrogen (mg/g fresh tissue)</th>
<th>Liver nitrogen (mg/g dry tissue)</th>
<th>Skin nitrogen (mg/g dry defatted tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>29.5±0.5</td>
<td>100.6±1.7</td>
<td>131.5±8.2</td>
</tr>
<tr>
<td>64</td>
<td>27.4±0.8</td>
<td>31.3±0.6</td>
<td>95.4±4.5</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>29.6±0.2</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td>28.8±0.7</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>27.9±0.4</td>
<td></td>
</tr>
<tr>
<td>147</td>
<td>26.4±1.0</td>
<td>26.5±1.4</td>
<td>94.6±2.2</td>
</tr>
</tbody>
</table>

L.S.D.(0.05) 2.27 11.9 19.1
L.S.D.(0.01) 3.04 15.9 25.7

Fig. 2. Total Amount of Hydroxyproline of Carcass and Skin and of N\(^{-}\)-Methylhistidine of Carcass in Control and Experimental Groups.

Each point represents the mean values of five rats. The vertical bars indicate standard errors. Rats in the experimental group were fed with the protein-free diet for 64 days and thereafter they were fed with the adequate protein diet for 83 days.

O-O, age control group; ⬤-●, experimental group.

A) Total amount of hydroxyproline of carcass. L.S.D.(0.05) = 0.50; L.S.D.(0.01) = 0.67.
B) Total amount of hydroxyproline of skin. L.S.D.(0.05) = 0.41; L.S.D.(0.01) = 0.55.
C) Total amount of N\(^{-}\)-methylhistidine of carcass. L.S.D.(0.05) = 2.52; L.S.D.(0.01) = 3.38.

methylhistidine in each group are also shown in Table IV. As shown in Fig. 2, the total hydroxyproline of the skin in the depleted rats was lowered to 72% of the initial control and 62% of age control value, respectively. It did not begin to increase immediately after refeeding with the adequate protein diet, but recovery could be found only in a later stage of refeeding. However, total hydroxyproline in the carcasses of protein depleted rats appeared to be slightly reduced as compared to the age control ones, but no significant difference between the two groups was found even at 5% level of probability. In the control group the total hydroxyproline of the carcass was increased with advancing age, whereas the value for the skin was approximately constant with age. The hydroxyproline concentrations of
Collagen and Muscle Protein in Protein Depleted and Repleted Rats

Table IV. Hydroxyproline and \( N^\gamma \)-Methylhistidine Concentration of Tissues in Both Control and Experimental Groups

The values are mean ± standard errors of five rats. The Least Significant Difference (L.S.D.) test is taken as the criterion of statistical significance with probability levels, 0.05 and 0.01.

<table>
<thead>
<tr>
<th>Days on diet</th>
<th>Carcass hydroxyproline (mg/g fresh tissue)</th>
<th>Skin hydroxyproline (mg/g dry defatted tissue)</th>
<th>Carcass ( N^\gamma )-methylhistidine (µg/g fresh tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>5.68 ± 0.29</td>
<td>—</td>
<td>107.0 ± 6.0</td>
</tr>
<tr>
<td>64</td>
<td>5.75 ± 0.73</td>
<td>9.16 ± 0.23</td>
<td>95.9 ± 2.8</td>
</tr>
<tr>
<td>72</td>
<td>—</td>
<td>7.04 ± 0.27</td>
<td>—</td>
</tr>
<tr>
<td>80</td>
<td>—</td>
<td>6.29 ± 0.15</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>6.69 ± 0.43</td>
<td>—</td>
</tr>
<tr>
<td>147</td>
<td>6.29 ± 0.51</td>
<td>6.90 ± 0.54</td>
<td>94.2 ± 4.8</td>
</tr>
</tbody>
</table>

L.S.D. \((0.05)\) 1.23 13.0 —
L.S.D. \((0.01)\) 1.64 17.4 —

Table V. Solubility Pattern of Skin Collagen

The values are mean ± standard errors of three to five rats, and are expressed as % of total skin collagen which is calculated from the hydroxyproline content by multiplying by a factor of 7.46.\(^\text{4)}\) The skin collagen was fractionated into the neutral salt-soluble, acid-soluble and insoluble collagen as described in Materials and Methods. The Least Significant Difference (L.S.D.) test is taken as the criterion of statistical significance with probability levels, 0.05 and 0.01.

<table>
<thead>
<tr>
<th>Days on diet</th>
<th>0.45 M NaCl-soluble (%)</th>
<th>0.5 M Acetic acid-soluble (%)</th>
<th>Insoluble (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>5.1 ± 0.6</td>
<td>—</td>
<td>11.7 ± 2.6</td>
</tr>
<tr>
<td>64</td>
<td>4.0 ± 1.1</td>
<td>0.6 ± 0.2</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>72</td>
<td>—</td>
<td>4.2 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>80</td>
<td>—</td>
<td>2.8 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>100</td>
<td>—</td>
<td>1.9 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>147</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>3.6 ± 1.1</td>
</tr>
</tbody>
</table>

L.S.D. \((0.05)\) 1.6 3.9 4.2
L.S.D. \((0.01)\) 2.1 5.3 5.7

both carcass and skin (Table IV) were markedly increased after 64 days of feeding with the protein-free diet, and they declined gradually after refeeding with the adequate protein diet. The results suggest that non-collagenous proteins were lost more rapidly and extensively than collagen from the tissues of rats fed with the protein-free diet. As shown in Fig. 2, the total amount of \( N^\gamma \)-methylhistidine in the carcass of depleted rats was markedly decreased (47% of the age control value), and then the value was rapidly increased by the refeeding with the adequate protein diet, but there was no significant difference in the concentration of the carcass \( N^\gamma \)-methylhistidine between the two groups (Table IV).

Solubility pattern of skin collagen

Table V shows the concentrations of the neutral salt-soluble, acid-soluble and insoluble collagens, expressed as percentages of the total collagen of the skin (calculated from the hydroxyproline content). The solubility pat-
tern of skin collagen in the control groups showed that with advancing age the concentrations of neutral salt-soluble and acid-soluble collagen were decreased, and the value for the insoluble collagen was increased. Neutral salt-soluble and acid-soluble collagen were also markedly decreased by protein depletion and rapidly recovered up to the 8th day of protein refeeding, but afterwards the salt-soluble collagen decreased gradually again with advancing age though the acid-soluble collagen did not.

**DISCUSSION**

In our previous experiment, adult rats during prolonged protein deprivation exhibited disturbance in behavior and hair loss around the mouth and neck, and one rat died on the 87th day of a protein-free diet. So, in the present study which was designed to study also the repletion process by refeeding with an adequate protein diet, the rats in the experimental groups were fed with a protein-free diet for 64 days. At 64 days of feeding with the protein-free diet, the animals lost approximately 50% of their body weights, 67% of the liver protein, 52% of the carcass muscle protein, 44% of the skin protein and 42% of the carcass protein compared to the age control.

A marked loss of skin collagen during prolonged protein deprivation was reported by Anasuya and Narasinga Rao and Angeleli et al. for the rat, and by Harkness et al. for the mouse. It has been also shown from the present study that the skin collagen decreased during protein depletion to 62% of age control group. On the other hand, changes of the amount of carcass (excluding skin) collagen during protein depletion are more complicated. Anasuya and Narasinga Rao and Mendes and Waterlow observed an actual increase in the total amount of carcass collagen in spite of the severe protein deficiency, which will be reported elsewhere. Moreover, it was shown in the experiment of Angeleli et al. in which young adult rats were used, that the amount of carcass collagen in the protein depleted rats was lower than that of age control group. This inconsistency with our present results may also be attributable to the age difference of rats used in both experiments.

When the animals in the protein deficient group were refed with the adequate protein diet, the body weight and total amount of proteins in each tissue rapidly increased and returned to the level of the initial control. The total amount of skin collagen did not increase during the early period of refeeding up to the 8th day, but thereafter it increased rapidly and returned to the initial control after 36 days of refeeding. No remarked increase in carcass collagen was observed during 36 days of refeeding, but the value built up gradually to the level of the age control after 83 days of refeeding (on day 147). At the end of the refeeding experiment, the concentrations (per unit tissue weight) of total nitrogen and hydroxyproline of each tissue of the experimental group did not differ from the values of the age control (Tables III and IV). In appearance, also, rats refed with the adequate protein diet provided with a protein-free diet. The age difference may account for this inconsistency, as pointed out by Angeleli et al. In the present study, as shown in Fig. 2, the amount of carcass collagen has been almost constant during protein depletion compared to the initial control and also there has been no significant difference statistically between the protein-free and age control groups despite some increase in the latter. It is not surprising that in the present study using adult rats, there has been no increase of carcass collagen accompanied with the growth of rats as shown by Anasuya and Narasinga Rao and Mendes and Waterlow. Our unpublished data for young rats also indicate an actual increase in the total amount of carcass collagen in spite of the severe protein deficiency, which will be reported elsewhere.
were not different from the age control except in body weight. These results suggest that the refeeding of depleted rats with the adequate protein diet brings about an almost complete recovery in liver protein, muscle protein and skin collagen which have been probably utilized as the main sources of amino acid pools for protein synthesis during prolonged protein deprivation.

It has been demonstrated by isotope experiments with $^{14}$C-labeled amino acids that there are different collagen pools with different turnover rates in the body of animals and that soluble collagen in any tissue has a shorter turnover time than insoluble collagen. Dawson and Milne reported that the amount of soluble collagen extracted from rat skin with neutral salt solution or acetic acid decreased with advancing age. Our results for the solubility pattern of skin collagen in the control groups also showed an increase of insoluble collagen with advancing age (Table V). Moreover, when the rats were fed with the protein-free diet for 64 days, the neutral salt-soluble and acid-soluble collagens drastically decreased. Prasad and Bose found that the gel reversibility and aldehyde content of neutral salt-soluble collagen were altered in the skin of protein depleted animals compared to the controls, indicating the impaired inter- and intra-molecular cross-linking and maturation of collagen. It therefore seems likely that protein deficiency causes not only a marked decrease in the amount of neutral salt-soluble collagen of the skin, but also an alteration of its physical and chemical nature.

Refeeding depleted rats with the adequate protein diet brought about a marked increase in the soluble collagen fractions of the skin. It should be noted that the soluble collagen of the skin increased rapidly during the early stage of refeeding up to the 8th day, but the total amount of skin collagen did not increase in this period and thereafter it began to increase in the later stage of refeeding. A marked decrease and increase in the soluble collagens during protein depletion and repletion may be interpreted as indicating an alteration of collagen synthesis in the tissue, since the soluble collagen is the first form of newly synthesized collagen and had a higher turnover rate than insoluble collagen.

On the other hand, several workers have supposed that skin collagen could be metabolized actively, but bone and other tissue collagens were metabolically inert. However, Neuberger and Slack reported that bone collagen was characterized by the presence of more than one metabolically distinct component, one of which had very rapid turnover (4 days). It may, therefore, be possible that the rate of catabolism and/or synthesis of carcass collagen is changed to some extent by protein depletion and repletion even if a marked change of the total amount of carcass collagen is not found.

REFERENCES

17) H. E. Firschein and J. P. Shill, Anal. Biochem., 14,