The effects of dietary nitrogen level on the collagen of rat skin

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(*Received* 9 *February* 1977 – *Accepted* 19 *July* 1977)

1. Male rats of approximately 120 g body-weight were maintained on a commercial stock diet containing 204 g crude protein (nitrogen × 6·25)/kg, a hydroxyproline-free high-protein (HP) diet containing 200 g casein/kg as the only protein source, or a low-protein (LP) diet containing 40 g casein/kg. After 6 weeks on these diets half of each group was transferred to a non-protein (NP) diet and the experiment was continued for a further 6 weeks. Animals from each group were killed at 4 d, 3 weeks and 6 weeks after the transfer to the NP diet.

2. Throughout the experiment the urinary excretion of N, hydroxyproline and creatinine, and the content and solubility of the skin collagen were determined.

3. When compared with a control group killed at the beginning of the experiment the rats maintained on the LP diet showed an increase of 25% in total N content of the skin but collagen content increased by 100%. Rats transferred from the HP to the NP diet lost both N and collagen from the skin, but those transferred from the LP to the NP diet lost N but increased the collagen content by 42%.

4. Protein deprivation brought about marked changes in the solubility of the skin collagen, suggesting an increase in the rate of maturation of skin collagen.

Several workers have reported appreciable losses of collagen, particularly from the skin of rats and mice, under conditions of protein deprivation (Harkness, Harkness & James, 1958, Čabak, Dickerson & Widdowson, 1963; Waterlow & Stephen, 1966; Anasuya & Narasinga Rao, 1970). In contrast Picou, Halliday & Garrow (1966), in a study of malnourished children, found that the amount of collagen continued to increase in spite of severe protein deficiency, and recently Love, Yamaguchi, Créach & Lavéty (1976) found that extra collagen is laid down in the skin and myocommata of cod during periods of starvation. Such conflicting results have given rise to the belief that species differences exist with regard to the lability of skin collagen (ARC/MRC Committee on Food and Nutrition Research, 1974). It is well established that, from a nutritional point of view collagen, because of its unbalanced amino acid composition, is a poor-quality protein and is unable to support growth in animals, so it would appear unlikely that body collagen could serve as a reserve protein for use by the animal during periods of protein deprivation. Collagen is, however, a major component of connective tissue and is essential for the maintenance of the structural integrity of the various organs and tissues. Thus any significant loss of body collagen would appear to be a somewhat disastrous reaction, or over-reaction, to nutritional stress with no apparent nutritional advantage to the animal. In view of these results and considerations we decided to re-examine the effects of protein deprivation on the collagen of rat skin, which together with the skeletal system, contains 60–70% of the total body collagen. Preliminary reports of this work have already been published (Dawson & Milne, 1976*a*, *b*).

EXPERIMENTAL

Animals and diets

Male rats from the Institute colony were used. From weaning the rats were maintained on a commercial stock diet containing 204 g crude protein (nitrogen × 6·25)/kg until they had reached a weight of 120–130 g, which they achieved at 7–8 weeks of age. At this point a group of six 'initial control' animals were killed. The remaining animals were divided into

two groups of twelve and twenty-four animals respectively. In the first experiment (Expt 1) the smaller group of twelve animals was continued on the commercial stock diet and the larger group of twenty-four animals was transferred to a low-protein (LP) diet containing 40 g casein/kg as the only protein source, as described by Palmer, McIntosh & Pusztai (1973). Experience had shown that at this level of protein intake the animals were just able to maintain body-weight. In a secone experiment (Expt 2) the smaller group of twelve animals was transferred to a hydroxyproline-free high-protein (HP) diet containing 200 g casein/kg as the only protein source and the larger groups of twenty-four animals was transferred to the LP diet as in Expt 1. After 6 weeks on these diets half of each group was transferred to a non-protein (NP) diet, the composition of which was identical to that of the LP diet except that the casein was replaced by an equal weight of maize starch. Thus for the final 6 weeks of the experiment the four dietary groups were: HP throughout, HP transferred to NP after 6 weeks (HP–NP), LP throughout and LP transferred to NP after 6 weeks (LP–NP). All diets were fed *ad lib.* In Expt 1 three animals from each of the HP and HP–NP groups were killed after 4 d of the NP diet and six animals from each of the LP and LP–NP groups. The remaining three animals from each of the HP and HP–NP groups, and the remaining six animals from each of the LP and LP–NP groups, were killed after 3 weeks on the NP diet. In Expt 2 a similar number of animals from each group were killed after 3 weeks and 6 weeks on the NP diet.

Collection of urine and urine analysis

Throughout the experiments one animal from each group was placed in a metabolism cage for 48 h in each week and urine was collected for the 48 h period. The urine samples were analysed for N, hydroxyproline and creatinine content. N and hydroxyproline were determined as described later, creatinine was determined by an automated procedure (Technicon Instruments Co. Ltd, 1967) using an AutoAnalyser (Technicon Instruments Co. Ltd, Basingstoke, Hants).

Removal of skins

Each animal was weighed, then killed by a blow on the head, and the fur was shaved from the whole of the body between the fore- and hind-limbs, using electric clippers. The shaved skin was removed. From this point all operations were carried out at 4°. The skin was spread out on an ice-cold glass plate, and the 'upper surface' was scraped free of any remaining fur with a scalpel or single-edged razor-blade. The 'under surface' was freed of any adhering fat or subcutaneous tissue in a similar manner. The shaved and scraped skin was weighed and chopped into small pieces with scissors. It was then deep-frozen until required.

Analysis of skin

Weighed samples of the chopped skin were taken for determination of total N content by the micro-Kjeldahl procedure and total hydroxyproline content by the method of Firschein & Shill (1966). Moisture content was determined by drying a sample of the chopped skin at 105° to constant weight.

Determination of hydroxyproline in urine

The hydroxyproline content of the urine samples was determined by the method of Firschein & Shill (1966). In order to check the reliability of this method the hydroxyproline content of several of the urine samples was determined by two other methods: i.e. the automated colorimetric procedure of Bannister & Burns (1970) and by ion-exchange chromatography of the urine samples using an automatic amino acid analyser (Locarte Co. Ltd, Emperors

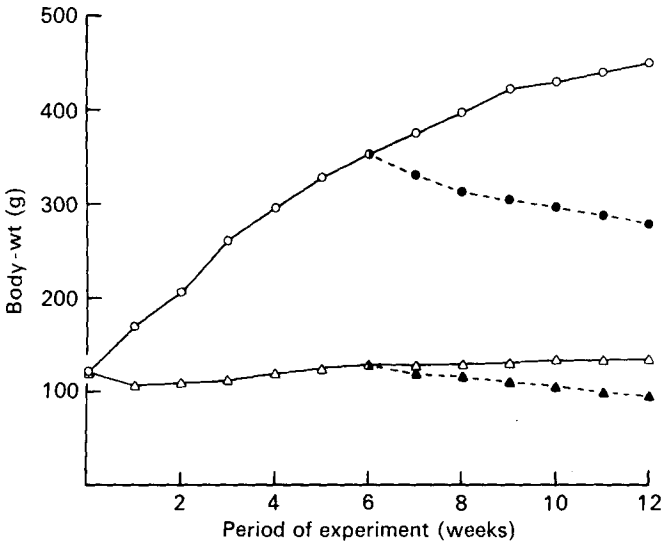

Fig. 1. Growth rate (g) of rats on high-protein (HP) and low-protein (LP) diets and the effect of transferring from these diets to a non-protein (NP) diet. (○—○), HP; (●—●), HP–NP; (△), LP; (▲), LP–NP; (- - -), period of NP feeding. For details of diets, see p. 181.

Gate, London). The results obtained by the three methods were in good agreement and differed by no more than 10%.

Solubility pattern of skin collagen

Collagen was exhaustively extracted from an approximately 1·5 g sample of chopped skin by sequential extraction in 0·45 M-sodium chloride, 1 M-NaCl and 0·5 M-acetic acid. Three extractions were carried out with each of the NaCl solutions and five extractions with the acetic acid solution. The extractions were carried out at 4° with constant stirring using a magnetic stirrer and each extraction was done for 48 h with 20 vol. solution. The material solubilized at each stage was removed by centrifuging at 30000 g for 45 min (Sorvall RC2B centrifuge; Ivan Sorvall Inc. Norwalk, Connecticut, USA) and the supernatant fraction was filtered through several layers of gauze. The three '0·45 M-NaCl' extracts were combined as were the three '1 M-NaCl' extracts and the five acetic acid extracts. The insoluble material remaining after these extractions was homogenized with distilled water to form a suspension and made to a known volume. The N and hydroxyproline contents of the extracts and of the insoluble suspension were determined as described previously.

RESULTS

The growth patterns of the rats on the various diets are shown in Fig. 1, where the points represent the mean weights of the animals in each group. For the first 6 weeks of the experiment the mean weights are those of the twelve animals in the HP group and of the twenty-four animals in the LP group. After 6 weeks, when the groups were subdivided, the mean weights are those of the six animals in each of the HP and HP–NP groups and of the twelve animals in each of the LP and LP–NP groups. After the first batch of animals from each group was killed (i.e. at 4 d in Expt 1, or at 3 weeks in Expt 2) the mean weights are those of the three animals remaining in each of the HP and HP–NP groups and of the six animals remaining in each of the LP and LP–NP groups. The growth of the animals

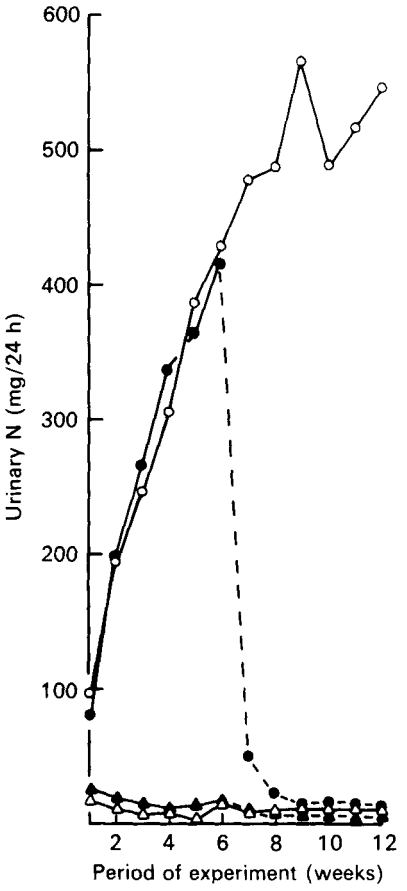

Fig. 2

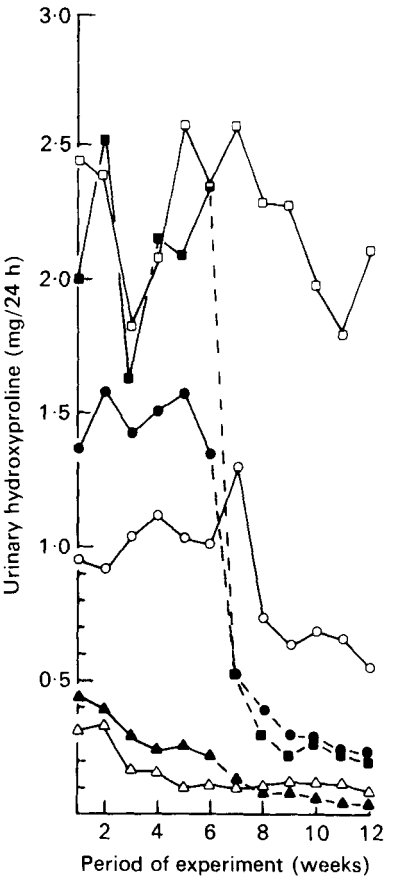

Fig. 3

Fig. 2. Urinary N excretion (mg/24 h) of rats on high-protein (HP) and low-protein (LP) diets and the effects of transferring from these diets to a non-protein (NP) diet. (○—○), HP; (●—●), HP–NP; (△), LP; (▲), LP–NP; (- - -), period of NP feeding. For details of diets, see p. 181.

Fig. 3. Urinary excretion of hydroxyproline (mg/24 h) by rats on high-protein (HP), low-protein (LP) and commercial stock diets and the effect of transferring from these diets to a non-protein (NP) diet. (○—○), HP; (●—●), HP–NP; (△), LP; (▲), LP–NP; (□), commercial stock diet; (■), commercial stock diet to NP; (- - -), period of NP feeding. For details of diets, see p. 181.

on the commercial stock diet and on the HP diet was virtually identical, so the curve for the HP group can be taken as referring to either the commercial stock diet or the HP diet. The HP group grew steadily throughout the experimental period of 12 weeks and the average weight of the animals increased from 122 g to 450 g.

The LP group lost weight during the first week after transfer to the LP diet but thereafter slowly gained weight until by the end of the experimental period, the average weight of the animals had increased from 122 g to 135 g. During the 6 weeks of NP feeding the rats previously accustomed to the HP diet lost about 70 g in weight and those previously accustomed to the LP diet lost about 30 g. All animals were active and no deaths occurred in any of the groups.

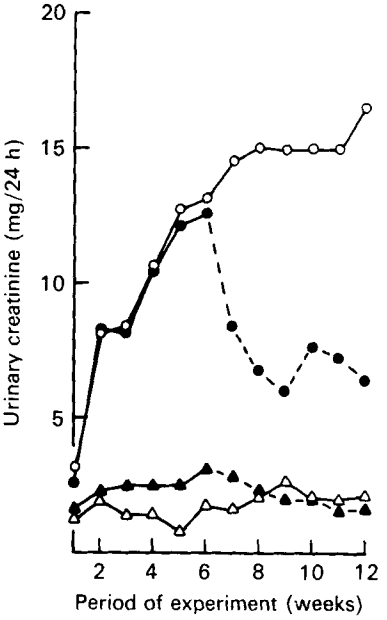

Fig. 4. Urinary excretion of creatinine (mg/24 h) by rats on high-protein (HP) and low-protein (LP) diets and the effect of transferring from these diets to a non-protein (NP) diet. (○—○), HP; (●—●), HP–NP; (△), LP; (▲) LP–NP; (- - -), period of NP feeding. For details of diets, see p. 181.

Urinary excretion of N, hydroxyproline and creatinine

The urinary excretion of N, hydroxyproline and creatinine by one rat from each of the dietary groups throughout the experimental period is shown in Figs. 2, 3 and 4 respectively. Only in the instance of urinary hydroxyproline excretion did the animals on the commercial stock diet differ significantly from those on the HP diet. The urinary excretion of both N and creatinine for animals on the HP diet shown in Figs. 2 and 4 can therefore be considered as referring to either the commercial stock diet or the HP diet. In Fig. 3 the urinary excretion of hydroxyproline by animals on both the commercial stock diet and HP diet is shown because these differed significantly (see p. 184).

From Fig. 2 it can be seen that in the animals maintained on the HP diet urinary N excretion increased rapidly throughout the experimental period but in the LP group it remained fairly constant and at a very low level (10–12 mg/24 h) throughout the experimental period. On transferring to the NP diet from the HP diet there was an immediate and dramatic decrease in urinary N excretion to a level very similar to that of the LP-fed animals. When the LP-fed animals were transferred to a NP diet urinary N excretion decreased by approximately 50% to approximately 5 mg/24 h.

Fig. 3 shows the pattern of urinary hydroxyproline excretion of animals on the various diets. As mentioned earlier, there was a significant difference between the animals on the commercial stock diet and those of the HP diet, the latter excreting only about half the amount of hydroxyproline excreted by those on the commercial stock diet. This was no doubt due to the fact that the commercial stock diet contained hydroxyproline-containing material, e.g. fish meal or meat-and-bone meal or both. Dietary hydroxyproline is not utilized in collagen synthesis and is largely excreted unchanged; the hydroxyproline content of collagen is derived from the hydroxylation of proline molecules after these have been incorporated into the newly-formed polypeptide chains. When animals from both the commercial stock diet and the HP diet were transferred to a NP diet there was an im-

Table 1. *Effects of dietary nitrogen level on the total N and total hydroxyproline content of rat skin and of feeding a non-protein (NP) diet* for 3 weeks and 6 weeks*

Group† 'Initial control'	Mean body-wt (g) 122		Mean skin wt (g) 5·02		Mean total N (mg/skin) 191		Mean total hydroxyproline (mg/skin) 71	
	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
HP	420	450	17·88	18·08	1017	1050	578	625
HP–NP	308	278	11·51	7·99	659	536	402	335
LP	131	135	4·33	4·60	209	248	119	141
LP–NP	110	96	2·83	2·52	148	166	86	101

HP, high-protein diet; LP, low-protein diet.
* For details of dietary regimens, see p. 181.
† For details of groups, see p. 181.

mediate and marked decrease in the excretion of hydroxyproline to almost identical levels, despite the large difference that existed before the transfer.

A further point of interest revealed by Fig. 3 is the decrease in hydroxyproline excretion in both the commercial stock diet and the HP-fed animals from the eighth week to the end of the experiment. This coincided with the age at which the animals attained sexual maturity and suggested that at this time there was a marked change in collagen metabolism. If this were so then the apparent marked decrease in hydroxyproline excretion due to the dietary changes was exaggerated by the physiological changes but nevertheless there is still a considerable contribution due to the dietary changes as shown in Fig. 3.

In the animals given the LP diet the urinary excretion of hydroxyproline steadily decreased for the first 5–6 weeks of the experimental period (from 0·3–0·4 mg/24 h to 0·1 mg/24 h) but from then it remained fairly constant. On transferring to the NP diet, urinary hydroxyproline excretion was reduced by approximately 50% (Fig. 3).

The pattern of urinary creatinine excretion is shown in Fig. 4. In the animals maintained on HP diets urinary creatinine increased to approximately 15 mg/24 h after approximately 7 weeks and appeared to 'level-off' at this value. Transferring animals to a NP diet resulted in a marked decrease in urinary creatinine excretion also. In the LP-fed animals urinary creatinine excretion fluctuated at approximately 2 mg/24 h throughout the experimental period and this was reduced by approximately 25% on transferring to the NP diet.

N and hydroxyproline content of skin

The total N and total hydroxyproline contents of the skins from the rats on the various diets together with the values for the 'initial control' group are shown in Table 1. The values for the initial control group are the means of the values obtained from the analyses of six individual skins. The other values are the means of three separate determinations. In the instance of the LP–NP group, two skins were pooled and minced together for each of the three determinations. The weight of skin was that of the shaved and scraped skin.

As would be expected with rapidly-growing animals the values for the HP group showed large increases in total N and total hydroxyproline content as compared with the 'initial control' group. The HP–NP group also contained much more N and hydroxyproline than the 'initial control' group, but the values obtained after 6 weeks of NP feeding show that the skin has lost both N and hydroxyproline compared with the values obtained after 3 weeks of NP feeding. Hydroxyproline is a measure of collagen content, since collagen contains virtually all of the hydroxyproline present in the animal body. Thus in this experi-

Table 2. *Solubility pattern of hydroxyproline in skin of rats on high-protein (HP) and low-protein (LP) diets followed by 4 d on non-protein (NP) diet*†

(Mean values for three rats or three × two rats/group)

	Group‡						Statistical significance of differences between groups:	
	'Initial control'	HP	HP–NP	LP	LP–NP	SE of means	HP, HP–NP *v.* LP, LP–NP	HP, LP *v.* HP–NP, LP–NP
Hydroxyproline concentration in whole skin (mg/g skin)	13·97	25·0	25·7	26·5	27·8	1·4	NS	NS
Hydroxyproline solubility (%) in								
0·45-M-sodium chloride	35·8	15·3	10·9	6·5	4·4	1·5	**	NS
1 M-NaCl	33·8	19·6	19·7	16·5	17·3	2·3	NS	NS
0·5 M-acetic acid	28·8	52·3	55·7	49·0	42·8	4·8	NS	NS
Insoluble hydroxyproline (%)	1·5	14·6	13·6	31·3	37·3	3·9	**	NS
Dry matter (g/kg)	355	394	370	362	371	17	NS	NS

NS, not significant.
** $P < 0.01$.
† For details of dietary regimens, see p. 181.
‡ For details of groups, see p. 181.

ment there is a loss of some 16% of the skin collagen during the third to sixth week of NP feeding. It is probable that similar, or greater, losses occurred during the first 3 weeks of NP feeding while the animals were adjusting to the NP diet. Harkness *et al.* (1958) reported losses of 16% of the skin collagen from mice on NP diets and Čabak *et al.* (1963) found a loss of approximately 25% of skin collagen from rats on NP diets, so the results reported here are in good agreement with those of other workers.

A very different situation emerges, however, when the results for the LP-fed animals are examined. Growth had been almost completely suppressed for 12 weeks and the animals were virtually the same weight as the 'initial control' group, but the skin contained 25% more N and 100% more collagen. In the LP–NP group skin weight was only approximately half that of the 'initial control' group and the total N was reduced by between 12 and 20%, but total collagen was 20–45% higher than in the 'initial control' group. However, skin N and skin collagen increased during the third to sixth week of NP feeding in contrast to the decrease found in the HP–NP group, and this is in agreement with the results of Picou *et al.* (1966).

Solubility pattern of skin collagen

The effects of HP and LP feeding and of NP feeding for 4 d, 3 weeks or 6 weeks on the solubility pattern of skin collagen are shown in Tables 2, 3 and 4. Table 2 also shows the solubility pattern of the skin collagen of the 'initial control' group, so that a comparison of the values for the 'initial control' group with the HP group shows the changes that occur in the solubility of collagen with age. The rats in the 'initial control' group were approximately 7 weeks old, whereas the rats in the HP group (Table 2) were approximately 14 weeks old. During this period from 7 to 14 weeks there is a considerable increase in the concentration of skin N and skin collagen, the latter increasing by more than 100%. In the younger rat almost 70% of the skin collagen is soluble in dilute NaCl solution and most of the remainder is soluble in dilute acetic acid; only 1·5% is insoluble. In the older rat

Table 3. *Solubility pattern of hydroxyproline in skin of rats on high-protein (HP) and low-protein (LP) diets followed by 3 weeks on non-protein (NP) diet*†

(Mean values for three rats or three × two rats/group)

	Group‡				SE of means	Statistical significance of differences between groups: HP, HP–NP *v.* LP, LP–NP	Statistical significance of differences between groups: HP, LP *v.* HP–NP, LP–NP
	HP	HP–NP	LP	LP–NP			
Hydroxyproline concentration in whole skin (mg/g skin)	30·98	35·10	26·90	30·37	0·9	**	***
Hydroxyproline solubility (%) in:							
0·45 M-sodium chloride	6·0	1·5	5·7	1·9	0·6	NS	***
1 M-NaCl	15·5	9·6	16·6	12·4	1·9	NS	*
0·5 M-acetic acid	39·6	44·9	47·9	32·2	4·6	NS	NS
Insoluble hydroxyproline (%)	32·7	38·3	24·7	37·4	7·5	NS	NS
Dry matter (g/kg)	423	411	431	395	8·0	NS	*

NS, not significant.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
† For details of dietary regimens, see p. 181.
‡ For details of groups, see p. 181.

there is a marked decrease in the NaCl-soluble fractions and an increase in the acid-soluble and acid-insoluble fractions.

The effects of transferring rats from HP or LP diets to a NP diet for 4 d are shown in Table 2. Thus if the HP group is compared with the HP–NP group, or the LP group is compared with the LP–NP group it is evident that the only collagen fraction showing any change was the 0·45 M-NaCl-soluble fraction which in both instances was reduced by approximately 30%. However, statistical analysis of these results shows that NP feeding for 4 d has no significant effect on collagen solubility (see Table 2). If the HP group is compared with the LP group or the HP–NP group is compared with the LP–NP group it is found that in both instances the 0·45 M-NaCl-soluble fraction has been reduced by almost 60% and the insoluble fraction has more than doubled as a result of LP feeding; Table 2 shows these differences to be significant ($P < 0.01$).

The results obtained after 3 weeks of NP feeding are shown in Table 3. At this point the differences in collagen solubility between the animals continuing to receive the HP and LP diets had disappeared but there was a significant difference ($P < 0.01$) in the concentration of collagen in the fresh whole skins. This could indicate that collagen metabolism had become completely adapted to the LP intake. In the animals deprived of protein, significant differences due to NP feeding were now apparent. There was a significant increase ($P < 0.01$) in the concentration of skin collagen, and significant reductions in the 0·45 M-NaCl-soluble and 1 M-NaCl-soluble fractions ($P < 0.001$ and $P < 0.05$ respectively).

NP feeding for 6 weeks (Table 4) resulted in more pronounced differences than at 3 weeks. The concentration of skin collagen was further increased, the 0·45 M-NaCl-soluble fraction was reduced by approximately 80%, the 1 M-NaCl-soluble fraction was reduced by approximately 60%, the acetic acid-soluble fraction was reduced by almost 50% and the insoluble fraction was increased by almost 100%. Again the only significant difference between the HP and LP groups was in the concentration of collagen in fresh whole skin.

Table 4. *Solubility pattern of hydroxyproline in skin of rats on high protein (HP) or low protein (LP) diets followed by 6 weeks on non-protein (NP) diet*†

(Mean values for three rats or three × two rats/group)

	Group‡				SE of means	Statistical significance of differences between groups: HP, HP–NP *v.* LP, LP–NP	Statistical significance of differences between groups: HP, LP *v.* HP–NP, LP–NP
	HP	HP–NP	LP	LP–NP			
Hydroxyproline concentration in whole skin (mg/g skin)	34·35	41·59	29·13	37·55	1·4	*	***
Hydroxyproline solubility (%) in:							
0·45 M-sodium chloride	4·26	0·90	3·37	0·90	0·2	NS	***
1 M-NaCl	14·5	6·08	14·8	6·08	0·9	NS	***
0·5 M-acetic acid	39·25	21·3	49·7	33·38	6·2	NS	*
Insoluble hydroxyproline (%)	34·3	67·3	29·2	53·3	7·4	NS	**
Dry matter (g/kg)	441	460	455	424	16	NS	NS

NS, not significant.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
† For details of dietary regimens, see p. 181.
‡ For details of groups, see p. 181.

Moisture content of skins

In all dietary groups the moisture content of the skins decreased throughout the course of the experiment giving an increase in dry matter (DM) content (Tables 2–4). With the exception of DM content in the NP groups after 3 weeks of NP feeding (Table 3) there was no significant difference in DM content between the groups.

DISCUSSION

When rats are subjected to acute or chronic protein deprivation there is a rapid physiological response which results in a marked suppression of the mechanisms concerned with the catabolism of body proteins and which is shown by the rapid decrease in the urinary excretion of N, hydroxyproline and creatinine. Thus within 1 week of transferring rats to HP and LP diets differences in these measurements were already evident and there was an almost immediate response when protein was suddenly removed from the diet, as shown in Figs. 2, 3 and 4. More detailed studies, however, revealed that the various mechanisms were affected to different extents and depended to some extent on the previous dietary history of the animals. Thus when rats previously accustomed to a HP or a LP diet were transferred to an NP diet, non-collagenous protein was lost from the skin of both groups, as shown in Table 1. The HP–NP group also lost collagen from the skin but in contrast the LP–NP group, despite the fact that they had lost approximately 25% of their weight and approximately 25% of the N from the skin, had increased the collagen content of the skin by 42% during the 12 weeks of the experiment. Our HP–NP group, which corresponds with the protein-free group of Harkness *et al.* (1958) showed similar losses of skin collagen to those reported by these workers. Similarly, our LP–NP group is probably comparable with the subjects studied by Picou *et al.* (1966) and in this instance our findings are in agreement with their findings.

Thus it would appear that in the rat the effects of protein deprivation on skin collagen depend on the previous dietary history of the animals. Presumably in the instance of the

HP–NP group a point would be reached after which no further losses of collagen would occur but losses of non-collagenous proteins would continue. Our results would suggest that, under similar conditions of protein deprivation, the collagen of rat skin reacts in the same manner as that of other species.

At the beginning of the experiment the rats transferred to the LP diet lost approximately 20 g in weight during the first week of LP feeding (Fig. 1) but then appeared to adapt to the diet and for the remainder of the experimental period gained 2–3 g/week. No doubt a similar period of adaptation would occur when animals were transferred to the NP diet after 6 weeks of experiment. Those animals transferred from the HP to the NP diet lost approximately 20 g during the first 4 d and those transferred from the LP to the NP diet lost approximately 7 g during the same period. At this point animals from each group were killed. The mean weight of skin from the HP and HP–NP groups differed by only approximately 0·2–0·3 g with a similar difference between the LP and the LP–NP groups. Virtually all of the loss in body-weight had therefore occurred from tissues other than the skin. Analysis of the skins (Table 2) showed that small but non-significant differences existed between the groups in hydroxyproline concentration and in DM content. Thus at this point there was no difference in collagen content between the HP and HP–NP groups nor between the LP and LP–NP groups. After 3 weeks on the NP diet the LP–NP group had a lower N content than the 'initial control' group but the collagen content had increased (Table 1), thus indicating a preferential loss of non-collagenous protein, but from 3–6 weeks of NP feeding both N and hydroxyproline contents increased. This increase in N and collagen content could be due to a limited synthesis of collagen from the breakdown products of non-collagenous body proteins and is in keeping with the findings of Picou *et al.* (1966) and supports our hypothesis that the animal must maintain its structural integrity at all costs. Nevertheless, it is possible that in the LP–NP group there could be a temporary loss of collagen between 4 d and 3 weeks after transfer to the NP diet while the animals are adapting to the NP diet.

As collagen matures it becomes less soluble owing to the formation of inter- and intramolecular cross-links (Verzar, 1964; Sinex, 1968; Bailey, 1969; Robins & Bailey, 1972) and this forms the basis of the extraction procedure now used. In our 'initial control' group, which were 7 weeks old at the time of slaughter, most of the skin collagen was soluble and only approximately 1·5% was insoluble. In rats at 14 weeks of age the concentration of skin collagen had doubled and the insoluble fraction had increased almost tenfold (Table 2). This illustrates the age-related changes that occur during the maturation of collagen. The processes involved in this maturation are not yet fully understood, and it is difficult to be precise in defining maturation, but for the purposes of the present work increasing insolubility is considered to indicate maturation. When animals were killed 4 d after the transfer to the NP diet the LP and LP–NP groups had insoluble fractions of at least 30% (Table 2) whereas the HP and HP–NP groups had insoluble fractions of less than 15%. In this sense, therefore, the collagen of the LP and LP–NP groups could be considered as more mature than that of the HP and HP–NP groups. Over the final 6 weeks of the experimental period the insoluble fraction of the LP group remained virtually unchanged (Tables 2, 3 and 4) so it would appear that this stable pattern had been established during the first 6 weeks of the experimental period and probably soon after the animals had adapted to the LP diet. In the HP group, on the other hand, there was an increase of well over 100% in the insoluble fraction between the '6 weeks and 4 d' point and the '9 weeks' point (Tables 2 and 3). From 9 weeks to 12 weeks of the experimental period no further change occurred in the insoluble fraction of the HP group (Tables 3 and 4) and, as in the LP group, this 'levelled-off' at approximately 30%. However, this extent of insolubility had been achieved in the LP group some weeks earlier than in the HP group. After 6 weeks on the NP diet

both the HP–NP and LP–NP groups showed an increase of almost 100% in the insoluble fraction compared with the HP and LP groups (Table 4). These results suggest, therefore, that protein deprivation increases the rate of maturation of the skin collagen.

Does this also suggest an increase in the physiological age of the animals? There are numerous reports regarding the correlation between diet and longevity and indicating that chronic underfeeding increases longevity in laboratory animals whereas dietary excesses or imbalances curtail lifespan (see Ross, Lustbader & Bras, 1976). It is possible that the increased rate of maturation of skin collagen found in this investigation could be due to a different type of maturation process involving different cross-links from those which develop during the normal maturation process. It has already been demonstrated that the collagen of the granuloma resulting from both acute and subacute inflammations possesses a different type of cross-link from that found in normal subcutaneous skin collagen, and during healing of the inflammation resorption of the granuloma occurs and the tissue reverts to its normal cross-linking pattern (Bailey, Bazin & Delaunay, 1973). However, Love *et al.* (1976) found that in the thickened skin and myocommata of starving cod the collagen prepared from the thickened tissues appeared to have identical properties to normal collagen except that the collagen from the myocommata appeared to have a higher extent of intermolecular cross-linking than that from normal myocommata. The skin collagen from the starving fish did not show this difference. McClain, Wiley & Beecher (1975) found that the aldehyde content of the α-1 chains of the skin collagen from rats on a LP diet (80 g protein/kg) was increased, thus increasing the potential for cross-linking, but paradoxically the yield of intramolecularly cross-linked β components was reduced by 48%. Thus the question as to whether more rapidly matured skin collagen differs from that matured normally remains to be resolved.

Transferring rats from both the HP and LP groups to a NP diet resulted in further marked changes in the solubility pattern of the skin collagen, both groups reacting in a similar manner. After 6 weeks of NP feeding the NaCl-soluble collagen was drastically reduced and the insoluble fraction was doubled when compared with animals maintained on the HP and LP diets. Again these results could be explained either by an increase in the rate of maturation or by a different type of maturation process. Alternatively this could simply be a reflexion of the very much reduced rates of synthesis and degradation of the skin collagen resulting in less dilution of the metabolic pool with newly-synthesized collagen.

It would be of interest to determine whether any of the changes brought about by LP or NP feeding could be reversed by repletion of the starved animals, and further studies will be carried out to elucidate these points.

The authors wish to thank Dr R. H. Smith for his helpful advice and discussions and Mr I. McDonald for the statistical analysis.

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Printed in Great Britain