Biomechanical and Biophysical Properties of Skin Collagen in Riboflavin or Pyridoxine Deficient Rats*

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Summary Skin collagen samples from vitamin B₂- or B₆-deficient rats, weight-matched controls, and control rats fed *ad libitum* were examined for biomechanical and biophysical properties like tensile strength, shrinkage temperature, gel-reversibility of salt-soluble collagen, and susceptibility of insoluble collagen to denaturing and proteolytic agents.

Deficiencies of riboflavin and pyridoxine, as well as food restriction, significantly reduced the tensile strength and shrinkage temperature and increased the gel-reversibility of collagen. The changes tended to be of greater magnitude in collagen from the vitamin-deficient rats. Solubilization of insoluble collagen by denaturing and proteolytic agents was markedly increased in collagen from riboflavin- or pyridoxine-deficient animals but not in that from food-restricted rats.

The results of this study support the hypothesis that the skin changes seen in riboflavin and pyridoxine deficiencies may be due to impaired collagen cross-linking and consequent alterations in the physical properties of the skin.

Key Words: tensile strength, skin collagen, riboflavin deficiency, pyridoxine deficiency, shrinkage temperature

Lysyl oxidase (EC 1.4.3.13) which is the key enzyme for initiating the synthesis of collagen cross-links is known to require copper and molecular oxygen as co-factors. Recent studies indicate that pyridoxal 5'-phosphate (PLP) may also be required for the activity of this enzyme [1,2]. Lysyl oxidase activity is markedly

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reduced in the cartilage and aorta of pyridoxine-deficient chicks, and the cross-link content is reduced in the skin of vitamin B₆-deficient rats [3,4]. Our earlier studies demonstrated that riboflavin deficiency also affects collagen content as well as maturity in rat skin. Thus the skin of both riboflavin- as well as pyridoxine-deficient rats has a higher proportion of soluble collagen and $\alpha:\beta$ sub-unit ratio in salt-soluble collagen. Food restriction showed similar trends but of lesser magnitude. The aldehyde content of the salt-soluble skin collagen from rats deficient in both these vitamins was reduced, but not that in food-restricted ones [5].

The changes in skin collagen observed in riboflavin deficiency may also be mediated through PLP-dependent lysyl oxidase, since the activity of the flavin enzyme pyridoxaminephosphate oxidase (EC 1.4.3.5) and the synthesis of PLP are affected in riboflavin deficiency [6–8]. In fact we have postulated that the molecular basis of skin lesions in vitamin B₂ and/or B₆ deficiency may be defective dermal collagen cross-linking [5]. A defective dermal collagen would render the overlying epithelial tissue weak and make it susceptible to stress and infection.

The present study was carried out to examine some biomechanical and biophysical properties of rat skin collagen in riboflavin and pyridoxine deficiencies, which may be expected to influence the structure and function of skin.

MATERIALS AND METHODS

One hundred and ten weanling male albino rats of the Wistar/NIN strain were divided into the following four groups: Group 1, controls fed *ad libitum*; Group 2, riboflavin-deficient; Group 3, pyridoxine-deficient; and Group 4, weight-matched-control group for riboflavin- and pyridoxine-deficient groups. Group 1 had 20 rats; and all others, 30 rats each. The animals were kept individually in screen-bottomed cages in a room maintained at a temperature of 22–25°C with a 12 h light, 12 h dark cycle.

The vitamin-deficient diets were prepared by deleting the appropriate vitamin from an otherwise complete diet containing 20 per cent casein, 70 per cent sucrose, 5 per cent peanut oil, vitamins, and minerals [9]. The weight-matched control rats were fed a restricted quantity of control diet so that their growth was comparable to that of the two vitamin-deficient groups. At the end of an experimental period of 7 weeks the animals were killed by cervical dislocation. The skin was removed and shaved off. The tail tendon was removed and stored at –20°C.

Tensile strength of the skin was measured using “Instron tensile tester” model 1112 [10].

A very thin fiber of collagen was removed from skin as well as from tendon, and the thermal shrinkage temperature of both was measured using a micro-linkage meter according to the method of Prasad and Bose [10].

At physiological temperature (37°C) collagen in dilute solution tends to form fibrils (gels). This results in an increase in optical density of the solution. On cooling to 4°C the fibrillar structure collapses, and this phenomenon (gel reversibility) is

enhanced by a reduction in collagen cross-linking. In the present experiment the percent reversibility of purified collagen gels on cooling from 37°C to 4°C was determined [10]. Salt-soluble collagen was purified as reported earlier [5]. After the extraction of salt-soluble collagen, the skin was shaken with 0.2 M citrate buffer, pH 3.5, to remove acid-soluble collagen. The remaining skin was thoroughly washed with water. This insoluble collagen was used to study the solubility of insoluble collagen in denaturing and proteolytic agents [10]. The reagents used were 6 M urea, 2 M potassium thiocyanate, and pronase (0.0016%). Pronase was obtained from Sigma Chemicals, St. Louis. All other chemicals used were of analytical grade.

RESULTS

Riboflavin as well as pyridoxine deficiency significantly reduced the tensile strength of the skin and shrinkage temperature of the skin and tail tendons. Food restriction produced similar changes, but of lesser magnitude (Table 1).

The reversibility of collagen gels on cooling from 37°C to 4°C was markedly greater in riboflavin- as well as pyridoxine-deficient animals. Food restriction had a similar effect, but of lesser magnitude (Table 1).

Both the vitamin deficiencies enhanced the solubility of insoluble collagen in denaturing and proteolytic such as urea (6 M), potassium thiocyanate (2 M), and pronase (0.0016%). Food restriction did not alter these properties (Table 2).

DISCUSSION

The changes in the physico-chemical properties of skin collagen, such as reduction in tensile strength and shrinkage temperature, the increase in temperature-dependent gel reversibility, and higher susceptibility to proteolytic and denaturing agents, in riboflavin- and pyridoxine-deficient rats are in agreement with the earlier observations on collagen solubility in non-denaturing agents and the $\alpha:\beta$ sub-unit ratio of soluble collagen [5]. These observations support our hypothesis that collagen cross-linking may be impaired in riboflavin, as well as in pyridoxine, deficiency. In the latter there is direct evidence to suggest that such a lesion may be due to a reduction in lysyl oxidase activity [3]. Direct evidence for the basic biochemical lesion in riboflavin deficiency is yet to come.

The marked reduction in the tensile strength of the skin suggests that the biochemical lesion has produced the critical point in terms of physical structure, and in the food-restricted as well as vitamin-deficient rats, the skin is structurally weakened and highly susceptible to stress. It may be pointed out that the caloric intake of the food-restricted, weight-matched control animals was less than 50 per cent of that of the vitamin-deficient groups. In the present study, a weight- and age-matched control group was used, since age as well as growth are known to alter collagen synthesis and maturation. The facts that the weight-matched control animals had a normal aldehyde content in the soluble-collagen fraction [5] and

that the susceptibility of their insoluble collagen to proteolytic and denaturing agents was the same as that of the *ad lib.*-fed control group (Table 2) may suggest that in food restriction the primary biochemical lesion may be beyond the lysyl oxidase step. Such a possibility has been indicated in the condition of protein deficiency [11].

The molecular mechanisms involved in the pathogenesis of various nutritional deficiency diseases are not understood. Changes in collagen content and properties have been observed in nutritional disorders where skin is affected. Examples of such disorders are protein calorie malnutrition in animals and humans, pellagra, scurvy, and deficiency of trace elements such as zinc [11–14].

There is however, a considerable degree of unexplained tissue and species specificity in collagen as well as elastin pathology. Thus, pyridoxine deficiency has been shown to affect elastin levels in chick aorta [15], but not in rat aorta [5]. Ribo-

Table 1. Effect of riboflavin or pyridoxine deficiency on biomechanical and biophysical properties of rat skin collagen.

	<i>Ad lib.</i> -fed control	Weight control	Riboflavin deficient	Pyridoxine deficient
Tensile strength of skin (kg/mm ²)	3.28±0.270 ^a (15)	1.09±0.042 ^b (15)	0.73±0.062 ^b (15)	0.87±0.077 ^b (15)
Shrinkage temperature of tail tendons (°C)	61.0±0.577 ^a (10)	57.6±0.670 ^b (10)	55.6±0.653 ^c (10)	55.5±0.342 ^c (10)
Shrinkage temperature of skin (°C)	62.6±0.267 ^a (10)	59.4±0.267 ^b (10)	54.6±0.371 ^c (10)	54.3±0.153 ^c (10)
Percent gel reversibility of salt (1 M NaCl)- soluble collagen	35.35±2.130 ^a (6)	51.33±6.399 ^b (6)	65.48±5.707 ^c (6)	69.7±5.520 ^c (6)

Values are means±SE. Numbers in parentheses indicate number of samples. Means in the row not sharing a common superscript (^a, ^b, or ^c) are significantly different ($p < 0.05$) by analysis of variance and Duncan's multiple range test.

Table 2. Effect of riboflavin or pyridoxine deficiency on susceptibility of insoluble collagen to denaturing and proteolytic agents.

	<i>Ad lib.</i> -fed control	Weight control	Riboflavin- deficient	Pyridoxine- deficient
Solubility of insoluble collagen by treatment with 6 M urea (% of total)	47.21±4.708 ^a	44.65±4.260 ^a	71.32±4.411 ^b	72.93±3.319 ^b
Solubility of insoluble collagen by treatment with 2 M KCNS (% of total)	45.0 ±0.855 ^a	41.83±2.968 ^a	76.46±2.379 ^b	74.46±7.224 ^b
Solubility of insoluble collagen by treatment with 0.0016% pronase (% of total)	42.95±2.418 ^a	39.48±3.405 ^a	70.92±5.455 ^b	66.45±3.669 ^b

Values are means±SE of six samples. Means in the row not sharing a common superscript (^a or ^b) are significantly different ($p < 0.05$) by analysis of variance and Duncan's multiple range test.

flavin deficiency also has no effect on contents of rat aorta elastin and cross-links such as desmosine and isodesmosine [5]. And copper deficiency has a greater effect on bone and aorta than on skin [16].

In the present study the skin samples were also examined histopathologically and electron microscopically. Such observations showed that food restriction as well as vitamin deficiency produced a considerable degree of epidermal and dermal atrophy. However, the magnitude of atrophy was greater in the vitamin-deficient animals. In pyridoxine deficiency the epidermis showed marked hyperkeratosis and evidence of inflammatory changes. Thus, despite having a similar magnitude of biochemical lesions in dermal collagen as riboflavin-deficient animals, the pyridoxine-deficient rat skin showed a greater degree of pathologic changes. Pyridoxine deficiency is known to affect the immune status of rats [17]. It is therefore possible that in these animals the already weakened skin becomes more susceptible to infections. The electron microscopic examination did not reveal any alterations in collagen structure.

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