

## EFFECT OF VITAMIN B6 DEFICIENCY ON THE CROSSLINK FORMATION OF COLLAGEN

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### 1. Introduction

With the recent advances in collagen chemistry it became apparent that the initial stage in the crosslink formation of collagen is the enzymic synthesis of aldehydes by removal of  $\epsilon$ -amino groups of certain lysine and hydroxylysine residues [1,2]. This enzyme named lysyl oxidase, one of the amine oxidases, has been purified to high specific activity and shown to be active upon both lysyl and hydroxylysyl groups in collagen and elastin [3,4]. It is well known that amine oxidases contain copper, and some of them require pyridoxal phosphate for their activity [5-7]. It can be inferred therefore that vitamin B6 is also required for enzymic activity of lysyl oxidase. This possibility has been reinforced by the findings that partial purification of lysyl oxidase from cartilage of chick embryos injected with [G-<sup>3</sup>H]pyridoxine hydrochloride revealed a single peak of radioactivity coinciding with a single peak of enzyme activity, and lysyl oxidase activity was inhibited after incubation with isonicotinic acid hydrazide [8].

In order to gain further information on this possibility, we have investigated aldehyde and crosslink formation of skin and bone collagens from vitamin B6-deficient rats.

### 2. Materials and methods

Wistar strain male rats averaging 50 g body wt were fed on a vitamin B6-deficient diet for 4 weeks [9]. The control diet was supplemented with 30 mg pyridoxine/100 g vitamin B6-deficient diet.

Vitamin B6 content in the brain and skin was

determined by an established microbiological method [10,11].

The skin and bone were cut into small pieces and powdered in liquid nitrogen. Following demineralization with 0.8 M EDTA (pH 7.5) the powdered bone was sequentially extracted with 1.0 M NaCl in 0.05 M Tris buffer (pH 7.4) and with 0.5 M acetic acid. The powdered skin was extracted with 1.0 M NaCl and with 0.15 M sodium citrate (pH 3.4). The residue obtained after these extractions, which is primarily insoluble collagen, was washed with distilled water and then lyophilized.

Following hydrolysis in 6 N HCl at 110°C for 24 h, hydroxyproline was determined [12] for the estimation of the collagen content in each extract and in the insoluble fraction.

Purified neutral salt soluble collagen from skin was completely dissolved in potassium phosphate buffer,  $\mu=0.4$  (pH 7.6) by stirring at 4°C for 24 h. This collagen solution and insoluble collagens from skin and bone were reduced with NaB<sup>3</sup>H<sub>4</sub> [13], they were then dialyzed extensively against 0.1 M acetic acid and lyophilized. The tritiated proteins were hydrolyzed in 3 N HCl at 107°C for 48 h, and the hydrolysates were dried. A portion of each hydrolysate was measured for specific radioactivity, and the data were expressed in terms of the measured hydroxyproline content of the hydrolysates. Chromatographic fractionation of the radioactive components of each hydrolysate was carried out on a 0.9 × 23 cm column of Aminex A-5 as in [14,15]. Aliquots were counted for radioactivity, and values under each crosslink peak were expressed as cpm/mg hydroxyproline in the hydrolysate loaded on a column.

### 3. Results and discussion

Prior to the chemical analysis of skin and bone collagens from rats fed on a vitamin B6-deficient and a control diet, the vitamin B6 contents of soft tissues, brain and skin, from both groups were determined. The vitamin B6 contents of these tissues were found to be significantly reduced after 4 weeks feeding with a vitamin B6-deficient diet (table 1).

Table 2 shows the solubilities of skin and bone collagens from vitamin B6-deficient and control groups. Although there were no significant changes in the amounts of citrate or acetic acid soluble collagens between the two groups, skin and bone collagens from the vitamin B6-deficient group were more soluble than those collagens from the control group when extracted with neutral salt solution.

The specific radioactivities of  $\text{NaB}^3\text{H}_4$ -reduced neutral salt soluble collagens from vitamin B6-deficient and control skin were compared in table 3. Collagen from vitamin B6-deficient skin, in solution, was found to incorporate only 27.6% (mean values) of

Table 1  
Vitamin B6 content in the brain and skin

	Control	B6-deficient
Brain	4728 ± 80 (100)	2547 ± 33 (53.9)
Skin	31.4 ± 1.3 (100)	18.0 ± 2.0 (57.3)

The data are expressed as  $\mu\text{g/g}$  wet wt of tissue. Figures in parentheses represent the % vitamin B6 content compared to the controls.

Table 2  
Changes in the solubility of skin and bone collagens

	Control		B6 deficient	
	Skin	Bone	Skin	Bone
1.0 M NaCl	11.0 ± 0.5	1.23 ± 0.09	23.8 ± 2.3	2.48 ± 0.07
0.15 M citrate	37.7 ± 1.9		39.4 ± 1.5	
0.5 M acetic acid		1.20 ± 0.09		1.51 ± 0.06
Total	48.7 ± 1.8	2.44 ± 0.14	63.2 ± 1.4	4.20 ± 0.18

The data are expressed as % total collagen content in each tissue.

Table 3  
Specific radioactivity of NaCl-soluble skin collagen

	Control	B6 deficient
$^3\text{H}$ cpm/ $\mu\text{g}$ hydroxyproline	218.3 ± 49.2 (100)	60.1 ± 5.8 (27.6)

Results are expressed as cpm/ $\mu\text{g}$  hydroxyproline in the hydrolysates. Figures in parentheses represent the % spec. radioact. compared to the control.

the radioactivity of collagen from control skin. This result indicates that collagen from the vitamin B6-deficient group contains an extremely low amount of aldehydes which participate in crosslink formation.

Table 4 shows the radioactivity recovered under each crosslink peak of insoluble skin and bone collagens. Both vitamin B6-deficient skin and bone collagens showed remarkably lower values under all crosslinks compared to the controls. The specific radioactivities of these insoluble collagens from vitamin B6-deficient group were also found to be lower than those of control collagens. The values were: control skin and bone collagens,  $214.5 \pm 10.0$  and  $177.0 \pm 17.5$  cpm/ $\mu\text{g}$  (hydroxyproline), vitamin B6-deficient skin and bone collagens,  $134.5 \pm 5.5$  and  $132.3 \pm 9.2$  cpm/ $\mu\text{g}$  (hydroxyproline).

The results from the crosslink studies and as well as the specific radioactivity analyses of skin and bone collagens clearly showed that vitamin B6 deficiency produces an extremely low amount of aldehyde crosslink intermediates and impairs the reducible crosslink formation. Thus, it may be apparent that lysyl oxidase requires vitamin B6 for its enzymic activity.

Table 4  
Formation of the reducible crosslinks

	Di-OH-LNL		OH-LNL		LNL	
	Control	B6-deficient	Control	B6-deficient	Control	B6-deficient
Skin	3168	2040	22 344	8100	20 856	6360
Bone	16 634	10 738	17 856	10 104	5784	2820

Results are expressed as cpm under each crosslink peak/mg of hydroxyproline in the hydrolysates loaded on a column

*Abbreviations* Di-OH-LNL, dihydroxylysnonorleucine, OH-LNL, hydroxylysnonorleucine, LNL, lysnonorleucine

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