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Regulation of collagen synthesis and maturation by 3,4-dehydroproline

Regulación de la síntesis y maduración de colágeno por 3,4-dehidroprolina

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Proline analogs are readily incorporated into collagen and noncollagen proteins. Since the imino acid content of collagen is greater than other proteins, it is suggested that the incorporation of a proline analog into cellular protein would have a maximal effect on collagen metabolism. Using a partially purified amino acyl tRNA synthetase preparation, various proline analogs were tested for their ability to inhibit Pro-tRNA synthesis. Amongst those tested, dehydroproline was the preferred inhibitor. Dehydroproline was also a substrate for amino acyl tRNA synthetase. When dehydroproline was added *in vitro* to membrane bound polysomes, the synthesis of collagenous proteins was preferentially inhibited.

The addition of dehydroproline to mammalian cell cultures caused a marked reduction in prolyl hydroxylase activity. Under these conditions growth of cells, activities of lysyl hydroxylase or lactic dehydrogenase were not affected. Reduction of prolyl hydroxylase activity by dehydroproline required protein synthesis. Removal of dehydroproline from the growth medium resulted in an increase in prolylhydroxylase activity.

Hepatic fibrosis can be induced in rats by chronic administration of carbon tetrachloride. Under these conditions, the collagen content and prolyl hydroxylase activity of the liver is enhanced. Treatment of these fibrotic animals with dehydroproline results in a reduction of prolyl hydroxylase activity of the liver. A mechanism by which dehydroproline reduces prolyl hydroxylase activity will be discussed. Since prolyl hydroxylase plays a key role in the maturation and deposition of collagen, specific inhibitors of this enzyme are potentially useful in controlling collagen deposition in various pathological conditions.

Collagen is the most abundant protein of the animal kingdom and constitutes about 25-30 percent of the total body protein. It is composed of three polypeptide chains referred to as α, or α_2 chains. Several reviews on the chemistry of collagen, have appeared (1-3).

Collagen is synthesized as a precursor, precollagen (pro-α chains) with peptide extensions at both the amino and carboxy termini (4). Subsequent to its synthesis, the molecule undergoes a variety of post-translational modifications such as hydroxylation of specific and lysyl residues of the ribosomal bound pro-α chains, disulfide bond formation and assembly of procollagen triple helix, transport of procollagen and extracellular cleavage of

the peptide extensions, crosslinking and formation of the insoluble collagen fibril (5).

Collagen has some unusual chemical characteristics (6). One third of all of the amino acid residues in the protein is glycine and approximately one-fourth of the residues are proline or hydroxyproline. This unusually large imino acid content of collagen has prompted several laboratories to study the effect of proline analogs on collagen metabolism. These studies (7-17) have demonstrated that the analogs of proline are readily incorporated into collagen and non-collagen protein of various biological systems. In this symposium, I will discuss *in vitro* and *in vivo* studies that we have carried out with 3,4-dehydroproline and its effect on collagen metabolism.

In order to choose an analog that would be most effective in replacing prolyl residues of cellular proteins, various proline analogs were assayed for their ability to inhibit ^{14}C-Pro-tRNA synthesis (18). Using a partially purified preparation of aminoacyl tRNA synthetase (19) and deacylated rabbit liver tRNA, L-3,4 dehydroproline was the most effective inhibitor (Table I). Dehydroproline was a competitive inhibitor of proline in this reaction (Ki = 100 μM). Dehydroproline was a substrate for the synthetase and the product of the reaction was dehydroprolyl-tRNA (Km DL-dehydroproline 550 μM; L-proline 45 μM). Using this assay system, it appears that dehydroproline is the analog that is most efficient in replacing prolyl residues of cellular proteins.

When dehydroproline was added to an *in vitro* polysomal system (20) active in procollagen synthesis, the incorporation of labeled glycine into procollagen was greatly decreased (19). Under these conditions the incorporation of glycine into non-collagen protein was only marginally reduced (Fig. 1). The incorporation of proline into both procollagen and non-procollagen protein was reduced, as expected of an analog. These observations support the view that dehydroproline is a relatively specific inhibitor of procollagen synthesis by polysomes. This effect may be due, in part, to a decrease in the rate of incorporation of ^{14}C-dehydroprolyl-tRNA into cellular proteins (19). Since collagen is enriched in prolyl residues as compared to other proteins, a decrease in the rate of incorporation of dehydroprolyl-tRNA into proteins would have a maximal inhibitory effect on procollagen synthesis and a marginal effect on non-procollagen protein. The degree of inhibition of non-procollagen proteins would depend on their proline content.

TABLE I

Effect of Various Proline Analogs on The Formation of Prolyl-tRNA By Use of a Partially Purified Aminoacyl-tRNA Synthetase[a]

	Prolyl-tRNA (pmoles)	*(%) Inhibition*
None	2.43	0
Cis-4-chloro-L-proline	1.55	36
DL-3,4-Dehydroproline	0.90	63
D-3,4-Dehidroproline	2.38	4
L-3,4-Dehydroproline	0.52	79
L-Azetidine-2-carboxylic acid	1.63	33
L-Thiazolidine-4-carboxylic acid	1.21	51
4,4-Difluoro-L-proline	1.49	39
Cis-4-hydroxy-L-proline	1.41	42

[a]The concentration of L-[^{14}C]proline in aminoacylation reaction was 51.6 μM. Analogs were present at a concentration of 1 mM. from Kerwar *et al.* (18).

Studies with mammalian cell cultures

When replicate cultures of 3T3 were exposed to ^{14}C-L-proline (25 μM) or ^{14}C-DL-dehydroproline (50 μM) the rate of incor-

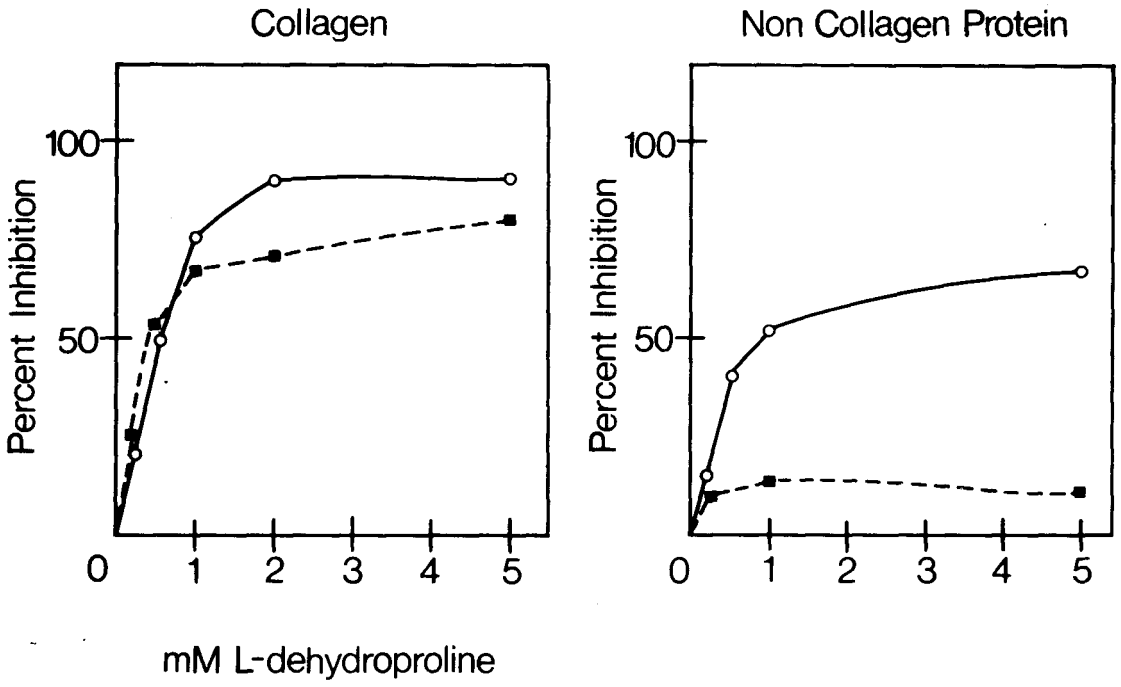

Fig. 1. Effect of L-3.4-dehydroproline on the incorporation of L-[^{14}C]proline (○----●) α f ∂ ^{3}H] glycine (■——■) into collagen and noncollagen proteins *in vitro*. From Kerwar *et al.* (18).

poration of ^{14}C-dehydroproline into cellular protein and collagen occured at one-fifth the rate observed for ^{14}C-proline (Fig. 2). These observations indicate that dehydroproline is incorporated into cellular proteins but at a decreased rate (21).

The effect of dehydroproline on the synthesis of collagen and non-collagen protein of 3T3 was examined (21). As shown in Figure 3, dehydroproline reduced the incorporation of glycine or lysine into collagen to a greater extent than the incorporation of these amino acids into non-collagen protein. These observations are consistent with the *in vitro* data observed with polysomes (Fig. 1). and support the view that dehydroproline inhibits collagen synthesis to a greater degree than non-collagen protein synthesis.

When replicate cultures of 3T3 cells were exposed to dehydroproline in the presence of ^{14}C-proline, the intracellular ^{14}C-hydroxyproline content was decreased (21). The extracellular ^{14}C-hydroxyproline was also decreased suggesting that dehydroproline may inhibit secretion of procollagen. However, the decrease in the hydroxyproline content by dehydroproline could be due to an inhibition in the incorporation of ^{14}C-proline and hence a decrease in the ^{14}C-hydroxyproline content (analog effect). It could also be due to an effect on prolyl hydroxylase, the enzyme responsible for the formation of hydroxyproline in collagen. To clarify this point, replicate cultures of L929 fibroblasts were exposed to various concentrations of dehydroproline for up to 5 days. Cells were harvested and assayed for protein (growth), prolyl hydroxylase, lysyl hydroxylase and lactic dehydrogenase. As seen in Table II, exposure of L929 cells to dehydroproline for 24 hrs resulted in a 70-80 percent reduction in the specific activity of prolyl hydroxylase whereas the specific activity of lysyl hydroxylase did not change significantly. During this period, cellular protein or the activity of lactic dehydrogenase were not affected. Exposure of cells to dehydroproline for 3 days resulted in a 60-80 percent reduction of prolyl hydroxylase, lysyl hydroxylase was reduced 30 percent. Even after 5 days, there was a 10-50 percent reduction in prolyl hydroxylase. These results indicate that one of the major effects of dehydroproline is a reduction in prolyl hydro-

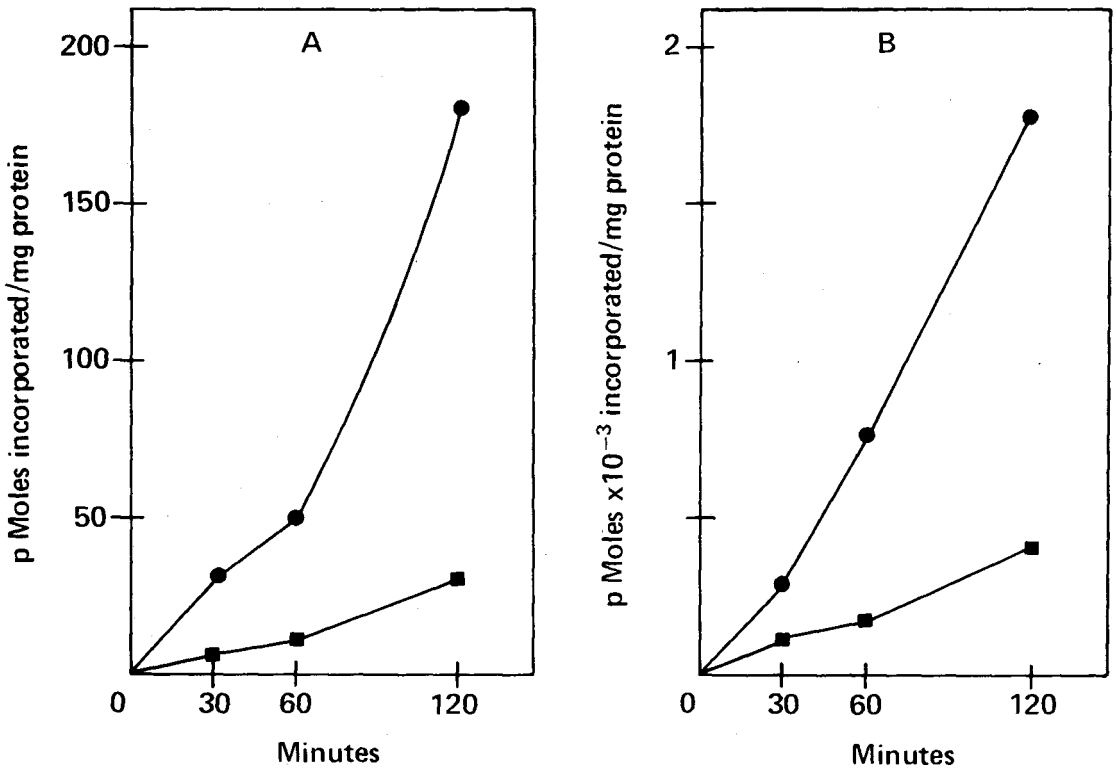

Fig. 2. Rate of incorporation of [^{14}C]-L-proline (○ – –○) and [^{14}C]-DL-3,4-dehydroproline (■ – –■) into (A) collagen and (B) into total protein of replicate, confluent 3T- cells. Each culture (approximately 5 × 10^6 cells/25 cm^2 dish) was incubated for varying periods of time in 10 ml of Eagle's medium containing either 12.5 μCi of [^{14}C]-DL-3,4-dehydroproline (50 μM) or 6.25 μCi of [^{14}C]-L-proline (25 μM). The pulse was terminated by the addition of 20 μg/ml cycloheximide. After trypsinization the cells were harvested and washed twice with 5 ml of phosphate buffered saline. The cells were disrupted by sonication in 1 ml of cold 20 mM Tris-Cl buffer, pH 7.4. From Kerwar *et al.* (21).

TABLE II

Effect of L-3,4-Dehydroproline on Growth, Lactic Dehydrogenase, Prolyl Hydroxylase and Lysyl Hydroxylase Activity in L929 Cells

	Control				*0.2 mM L-3,4-Dehydroproline*				*0.1 mM L-3,4-Dehydroproline*				*0.05 mM L-3,4-Dehydroproline*			
Day	*Protein* mg	*PH*	*LH*	*LDH*	*Protein* mg	*PH*	*LH*	*LDH*	*Protein* mg	*PH*	*LH*	*LDH*	*Protein* mg	*PH*	*LH*	*LDH*
0	1.5	34.1	1.76	—	1.5	34.1	1.76	—	1.5	34.1	1.76		1.5	34.1	1.76	
1	2.8	28.8	0.65	6.2	2.6	5.9	0.76	6.9	2.8	7.6	0.58	5.8	2.5	8.3	0.59	6.0
3	7.0	25.1	0.53	6.5	6.6	5.8	0.37	8.1	7.2	8.4	0.44	7.2	7.4	9.1	0.66	8.5
5	13.0	40.5	0.71	7.9	11.7	21.0	0.70	8.6	14.0	23.8	0.58	7.1	14.2	35.5	0.75	9.5

Replicate flasks were seeded with 5.4 × 10^6 cells per 75 cm^2 flask in 10 ml of Eagle's medium. The cultures were incubated at 37°; the medium was replaced daily. Cells were harvested by trypsinization and washed twice in phosphate buffered saline before determining the specific activities or prolyl hydroxylase (PH), lysil hydroxylase (LH) and lactic dehydrogenase (LDH) as described. The activity of LDH is expressed as the change in optical density at 340 nm/mg protein/min, while the activity of PH and LH is expressed as cpm × 10^{-3}/mg protein.
From Kerwar *et al.* (21).

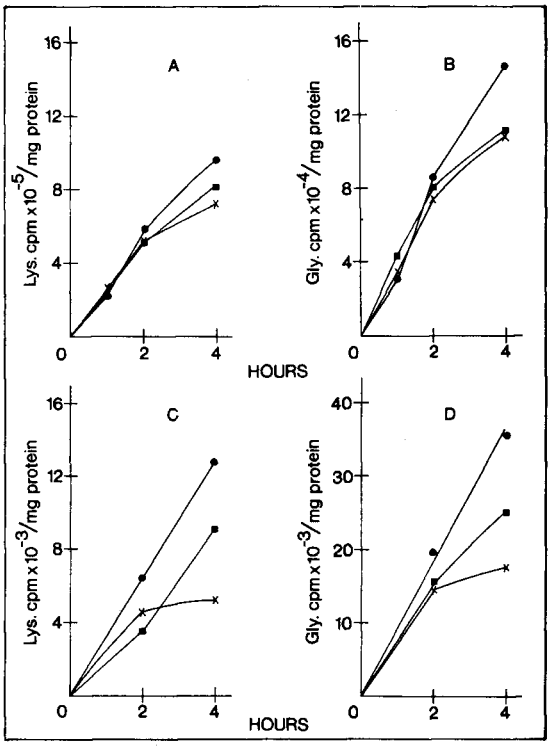

Fig. 3. Effect of L-3,4-dehyproproline on the incorporation of [^{3}H]-L-lysine and [^{14}C]-L-glycine into total protein (A and B) and into collagen (C and D) of replicate, confluent 3T3 cells. Each culture (6 × 10^6 cells/75 cm^2 dish) was pulsed with 166 μCi of [^{3}H]-L-lysine (0.04 M) and 4.2 μCi of [^{14}C]-L-glycine (4.3 μM) in 10 ml Eagle's medium containing various concentrations of L-3.4-dehydroproline (1 mM ■——■; 2 mM (x——x) or no additions (Control (●——●). from Kerwar *et al.* (21).

xylase activity, lysyl hydroxylase may be marginally reduced but the proliferation of cells or the activity of lactic dehydrogenase are not affected. A reason for the decreased sensitivity of prolyl hydroxylase of L929 cells after a five day exposure to dehydroproline has been suggested (21).

The time course of reduction of prolyl hydroxylase by L-dehydroproline has been investigated (21). At a concentration of 0.2 mM, prolyl hydroxylase is reduced 50-60 percent. D-dehydroproline has no effect and DL-dehydroproline is one-half as effective as the L isomer.

Dehydroproline does not inhibit the activity of prolyl hydroxylase when it is added in vitro and as shown in Table III, protein synthesis is

TABLE III

Requirement of Protein Synthesis for Reduction of Prolyl Hydroxylase Activity by L-3,4-Dehydroproline

Addition to Grow Medium	*Specific Activity cpm × 10^{-3}*	*Percent Inhibition*
None	39.7	0
L-3,4-Dehydroproline	12.0	70
Cycloheximide	32.9	17
L-3,4-Dehydroproline + Cycloheximide	42.4	0

Confluent cultures of L929 cells (2 × 10^6 cells/25 cm^2 dish) were exposed to either L-3,4-dehydroproline (0.2 mM), cycloheximide (10 μg/ml) or both for 6 hr. Then, cells were harvested and assayed for prolyl hydroxylase activity.
From Kerwar *et al.* (21).

required for the reduction of prolyl hydroxylase *in vivo*. Cycloheximide, an inhibitor of protein synthesis blocks the effect of dehydroproline.

The reduction of prolyl hydroxylase activity by dehydroproline is reversible. Replicate cultures of L929 cells were exposed to various concentrations of L-dehydroproline for 24 hours. During this period the specific activity of prolyl hydroxylase was reduced 60-80 percent. At the end of 24 hours, medium containing dehydroproline was removed and the cultures were replenished with fresh medium containing 5 mM L-proline. After an additional 48 and 96 hours of growth, the cells were harvested and assayed for prolyl hydroxylase activity. As shown in Table IV, removal of dehydroproline from the growth resulted in a time dependent increase in the specific activity of prolyl hydroxilase and after 96 hours the specific activity of prolyl hydroxylase was approximately 80-90 percent of the control activity. These observations suggest that the reduction of prolyl hydroxylase *in vivo* by dehydroproline is reversible.

Animal experiments with dehydroproline

Recent studies by Salvador *et al.* (22) have shown that L-dehydroproline is a fairly specific and effective inhibitor of collagen synthesis and of prolyl hydroxylase in the uterus.

TABLE IV

Recovery of Prolyl Hydroxylase Activity of L929 Cells After Removal of L-3,4-Dehydroproline

		Prolyl Hydroxylase Activity $cpm \times 10^{-3}$/mg *protein*		
Growth		*Concentration of L-3,4-Dehydroproline (mM)*		
Day	*Control*	*0.2*	*0.1*	*0.05*
0	34.1	34.1	34.1	34.1
1	28.8	5.9	7.6	8.3
3	25.0	13.6	16.2	17.1
		(5.8)	(8.4)	(9.1)
5	40.4	31.7	37.6	37.2
		(21.0)	(23.8)	(35.5)

Replicate flasks were seeded on Day 0 with 5.4×10^6 cells per 75 cm^2 dish in 10 ml Eagle's medium containing either 0.2, 0.1 or 0.05 mM L-3,4-dehydroproline. After 24 hr (Day 1), the growth medium containing L-3,4-dehydroproline was removed and replaced with Eagle's medium containing 5 mM L-proline. The values in parentheses show the specific activity of prolyl hydroxylase when L-3,4-dehydroproline was present in the growth medium throughout the duration of the experiment.
From Kerwar *et al.* (21).

These observations have been extended to a rat hepatic fibrotic model (23). Chronic administration of carbon tetrachloride to rats causes an increase in prolyl hydroxylase activity and in collagen content of the liver (24). When these animals are administered DL-dehydroproline for 5 days (100 mg/kg), the specific activity of prolyl hydroxylase of the liver and lung is markedly reduced (Table V and VI). These observations indicate that dehydroproline is effective in reducing prolyl hydroxylase activity in animal models where an increase in enzyme activity has been induced.

Role of prolyl hydroxylase in the stability of the collagen triple helix

Studies by Rosenbloom and by Prockop (25, 26) have indicated that the hydroxyproline residues of collagen are required for the stability of the collagen triple helix. Collagen with the normal amount of hydroxyproline residues is triple helical at body temperature and is resistant to proteolysis by nonspecific proteases. Collagen, deficient in hydroxyproline is not triple helical at body temperature, and in the random coil configuration is readily digested by nonspecific proteases. Therefore, collagen in a nonhelical configuration is not deposited in the tissue but is degraded. Compounds that are specific inhibitors of prolyl hydroxylase which lead to the synthesis of hydroxyproline deficient collagen can modulate collagen deposition *in vivo*. If these compounds are nontoxic with minimal side effects, and are specific, these are potentially useful in the treatment of a variety of disease states where excessive collagen deposition occurs. The studies reported in this symposia indicate that dehydroproline *in vivo* reduces prolyl hydroxylase activity. Whether dehydroproline can be used clinically will depended on its complete toxicological and pharmacological properties.

Mechanisms of reduction of prolyl hydroxylase and dehydroproline

The mechanism by which dehydroproline reduces prolyl hydroxylase has not been established. Rosenbloom and Prockop (10) have indicated that the dehydroprolyl residue in collagen is not hydroxylated by prolyl hydroxylase. These studies have suggested that prolyl hydroxylase binds to collagen containing dehydroproline in an irreversible manner. This enzyme-quasi substrate complex does not disassociate and hence a reduction in enzyme activity is observed. McGee *et al.* (27) have shown that dehydroprolyl bradykinin is a competitive inhibitor of prolyl hydroxylase. Other mechanisms by which dehydroproline *in vivo* can cause a reduction in prolyl hydro-

TABLE V

Effect of DL-3,4-Dehydroproline on Prolyl Hydroxylase and Lactic Dehydrogenase Activities of the Liver[a]

Group	*Specific Activity*	
	Prolyl hydroxylase (cmp/mg of protein × 10^{-3})	*Lactic dehydrogenase (Δ OD Units/mg of protein/min)*
Control	3.36 ± 0.14	6.86 ± 0.46
Control + dehydroproline	2.23 ± 0.29*	10.77 ± 0.74***
CCl_4	12.56 ± 1.56	—
CCl_4 + dehydroproline	4.26 ± 0.14**	—

[a]Control group (10 animals) refers to those treated with mineral oil and the CCl_4 group (10 animals) refers to those treated with CCl_4. After 12 weeks of treatment, five animals from either the control or the CCl_4 group were treated with dehydroproline (100 mg/kg) for 5 consecutive days.

*Significantly different from control: $p < 0.02$.

**Significantly different from CCl_4 livers: $p < 0.001$.

***Significantly different from control: $p < 0.01$.

From Kerwar *et al.* (24).

TABLE VI

Effect of DL-3,4-Dehydroproline on Prolyl Hydroxylase And Lactic Dehydrogenase Activities of The Lung[a]

Group	*Prolyl hydroxylase (cpm/mg of protein × 10^3)*	*Lactic dehydrogenase (Δ OD Units/mg of protein/min)*
Control	10.75 ± 0.19	2.28 ± 0.1
Control + dehydroproline	7.47 ± 0.65[b]	2.24 ± 0.23

[a]The details of the experiment are the same as those described in footnote a, Table V.

[b]Significantly different from control: p 0.01.

From Kerwar *et al.* (24).

xylase have been suggested (21). Further experiments are required before we can understand the mechanism by which dehydroproline causes a reduction in prolyl hydroxylase.

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