Effect of Cortisol Acetate on Collagen Biosynthesis and on the Activities of Prolyl Hydroxylase, Lysyl Hydroxylase, Collagen Galactosyltransferase and Collagen Glucosyltransferase in Chick-Embryo Tendon Cells

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Collagen synthesis and the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase were studied in isolated chick-embryo tendon cells after the administration of cortisol acetate to the chick embryos. When the steroid was injected 1 day before isolation of the tendon cells, collagen synthesis was decreased, even though the enzyme activities were not changed. When cortisol acetate was given as repeated injections over a period of 4 days, both collagen synthesis and the enzyme activities decreased. The hydroxylase activities decreased even more than the two collagen glycosyltransferase activities, both in isolated cells and in whole chick embryos. The amount of prolyl hydroxylase protein diminished to the same extent as the enzyme activity, indicating that cortisol acetate inhibits enzyme synthesis. The inhibitory effect of cortisol acetate on collagen synthesis and on the enzyme activities was partially reversible in 3 days. Total protein synthesis was completely restored within this time. Only massive doses of cortisol acetate inhibited collagen synthesis *in vitro*. Additional experiments indicated that cortisol acetate did not decrease the rate of the enzyme reactions when added directly to the enzyme incubation mixtures. The results suggest that cortisol acetate decreases collagen synthesis both by its direct effect on collagen polypeptide-chain synthesis and by decreasing the activities of enzymes involved in post-translational modifications.

The biosynthesis of collagen involves a number of post-translational modifications of the initial polypeptide chains. Some of these modifications take place before procollagen is secreted from the cells into the extracellular matrix and some occur after the secretion. The intracellular modifications consist of the hydroxylation of appropriate proline and lysine residues to hydroxyproline and hydroxylysine residues, the glycosylation of appropriate hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine residues, chain association, disulphide bonding and formation of the triple helix (for reviews on collagen biosynthesis, see Bornstein, 1974; Martin *et al.*, 1975; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). The enzymes prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase are needed in these reactions (Kivirikko & Risteli, 1976; Prockop *et al.*, 1976).

Anti-inflammatory steroids have several effects on collagen metabolism (Kivirikko, 1970; Uitto & Mustakallio, 1971). More recent results indicate that they inhibit collagen synthesis specifically (Cutroneo & Counts, 1975) and that the activity of prolyl hydroxylase is decreased after treatment with anti-inflammatory steroids (Cutroneo *et al.*, 1975; Oikarinen, 1977). No data are available,

however, on the activities of lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase.

In the present study, collagen synthesis and the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase were measured in isolated chick-embryo tendon cells after the administration of cortisol acetate to the chick embryos. Enzyme activities were also assayed in whole chick embryos and the amount of immunoreactive prolyl hydroxylase protein was determined in the tendon cells. The aim of the present study was to elucidate whether these enzyme activities change in a similar manner and whether changes in the enzyme activities correlate with those in collagen synthesis and with the extent of the post-translational modifications in this protein.

Experimental

Materials

Fertilized eggs of white Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and were incubated in a moist atmosphere at 37°C until used.

[¹⁴C]Proline and [¹⁴C]lysine were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.),

purified bacterial collagenase was from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.), and trypsin [2.5% (w/v) solution in 0.9% NaCl] was from Gibco Corp. (Grand Island, NY, U.S.A.). Foetal calf serum was purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.) and cortisol acetate from Yliopiston Apteekki (Helsinki, Finland).

Isolation and incubation of tendon cells

Cortisol acetate dissolved in 0.2 ml of 0.9% NaCl was pipetted on to the chorioallantoic membrane in the concentrations indicated in the legends to the Tables. Control eggs received the same amount of 0.9% NaCl. After the periods indicated, the tendon cells were isolated by controlled digestion with collagenase and trypsin (Dehm & Prockop, 1971). The cells were counted with a haemocytometer, and some were used for the assay of the enzyme activities and some incubated in a modified Krebs medium (Dehm & Prockop, 1971) without the addition of cortisol acetate containing [^{14}C]proline or [^{14}C]lysine for 2 h as indicated. After the incorporation period, synthesis of protein and collagen was stopped by adding cycloheximide and $\alpha\alpha'$ -bipyridyl to give a final concentration of 100 μg of cycloheximide and 1 μmol of $\alpha\alpha'$ -bipyridyl/ml (Dehm & Prockop, 1971).

In the experiments on the effect of cortisol acetate on collagen synthesis *in vitro*, tendon cells isolated as described above were incubated with or without cortisol acetate or in a modified Krebs medium (Dehm & Prockop, 1971) containing [^{14}C]proline, and the samples handled as described above after incubation for 2 h.

Preparation of collagen substrates

Denatured [^{14}C]proline- and [^{14}C]lysine-labelled protocollagen substrates were prepared as described previously (Risteli & Kivirikko, 1976; J. Risteli *et al.*, 1976).

Collagen for the assay of collagen galactosyltransferase and collagen glucosyltransferase activities was prepared from calf skin (Myllylä *et al.*, 1975a) and denatured before the assay (Myllylä *et al.*, 1975b; L. Risteli *et al.*, 1976).

Assay of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities in tendon cells and in whole embryos

Tendon cells were isolated as described above. The cells were homogenized in duplicate in a solution containing 0.1 M-glycine, 0.1 M-NaCl, 50 μM -dithiothreitol, 20 mM-Tris/HCl buffer (pH 7.5 at 4°C) and 0.1% (w/v) Triton X-100 with 60 strokes of a Teflon-glass homogenizer (Risteli & Kivirikko, 1974; Kao *et al.*, 1975). The homogenates were incubated at 4°C for 60 min and then centrifuged at 15000g for 30 min at 4°C (Risteli & Kivirikko, 1974). The protein content of the 15000g supernatant was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard.

For the enzyme assays in whole chick embryos, the embryos were frozen and homogenized after pulverization with a Teflon-glass homogenizer in the homogenizing medium described above. The 15000g supernatants of the homogenates were used for the determination of protein content and enzyme activities.

Prolyl hydroxylase and lysyl hydroxylase activities were assayed with 60000 d.p.m. of [^{14}C]proline- or 110000 d.p.m. of [^{14}C]lysine-labelled protocollagen substrate (Kivirikko & Prockop, 1972; Tuderman *et al.*, 1975a). Substrates of various specific radioactivities were used here in the enzyme assays, so that comparisons of the enzyme activities can only be made within each Table.

Collagen galactosyltransferase and collagen glucosyltransferase activities were assayed as described previously (Myllylä *et al.*, 1975a; Anttinen, 1976), except that 30 μM -UDP-[^{14}C]galactose (specific radioactivity 15 Ci/mol) was used for the galactosyl-

Table 1. *Effect of cortisol acetate on the incorporation of [^{14}C]proline and formation of hydroxy[^{14}C]proline in isolated tendon cells*

Two injections of cortisol acetate (200 μg /embryo) were given at an interval of 3 h, as described in the Experimental section. The cells were isolated after incubation for 24 h, and 10×10^6 cells were incubated with 1 μCi of [^{14}C]proline in 2 ml of a modified Krebs medium for 2 h. Total radioactivity and hydroxy[^{14}C]proline were assayed as described in the Experimental section. The values are the means of duplicate samples from one experiment.

Cells	Total ^{14}C radioactivity		Total hydroxy[^{14}C]proline	
	($10^{-4} \times \text{d.p.m.}$)	Change (%)	($10^{-4} \times \text{d.p.m.}$)	Change (%)
Control	9.6		3.2	
Cortisol-treated	6.1	-36	1.7	-47

transferase activity assay and 60 μ M-UDP-[¹⁴C]-glucose (specific radioactivity 6.6Ci/mol) for that of glucosyltransferase activity.

The amount of immunologically cross-reacting prolyl hydroxylase protein was measured with a specific radioimmunoassay (Tuderman *et al.*, 1975b). Crude chick-embryo enzymes produced by (NH₄)₂SO₄ fractionation (Myllylä *et al.*, 1976; L. Risteli *et al.*, 1976) were used in the experiments on the direct effect of cortisol acetate on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase.

Other assays

After incubation with [¹⁴C]proline, the medium and cell fractions were dialysed against running tap water for 24–48 h and the retained materials were hydrolysed in sealed tubes in 6M-HCl at 120°C overnight. The hydrolysed samples were evaporated over a steam bath and dissolved in 4ml of water. Hydroxy[¹⁴C]proline content was assayed by a specific radiochemical procedure (Juva & Prockop, 1966).

After incubation with [¹⁴C]lysine, the medium and cell fractions were dialysed against running tap water for 24–48 h. The samples were evaporated on a steam bath and hydrolysed with 2.0ml of 2M-NaOH in sealed disposable glass ampoules at 105°C for 24 h. After hydrolysis the samples were handled as described previously (Myllylä *et al.*, 1975a; Oikarinen *et al.*, 1976). A small portion was taken for the assay of total radioactivity in the sample and the rest was assayed in an amino acid analyser.

Amino acid analysis was carried out in a 63 cm column (Jeol 5AH amino acid analyser) as described previously (Askenasi, 1973; Oikarinen *et al.*, 1976). Fractions (about 2ml) were collected every 2 min, dissolved in 5ml of Instagel (Packard Instrument Co., La Grande, IL, U.S.A.), and assayed in a liquid-scintillation counter (see Oikarinen *et al.*, 1976).

All ¹⁴C radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 80% and a background of 25c.p.m. by using the scintillants reported in the assay procedures (Myllylä *et al.*, 1975a; Oikarinen *et al.*, 1976).

Results

Incorporation of proline and lysine and synthesis of hydroxyproline, hydroxylysine and glycosylated hydroxylysine by tendon cells after treatment of the embryos with cortisol acetate

The cells were isolated in all the experiments when the chick embryos were 17 days old. In the first experiments the cortisol acetate was injected twice at an interval of 3 h on to the chorioallantoic membranes,

Table 2. Effect of cortisol acetate on the incorporation of [¹⁴C]proline and [¹⁴C]lysine and formation of hydroxy[¹⁴C]proline, hydroxy[¹⁴C]lysine and glycosylated hydroxy[¹⁴C]lysine in isolated tendon cells

Cells	Total [¹⁴ C]proline		Total hydroxy[¹⁴ C]proline		Total [¹⁴ C]lysine		Total hydroxy[¹⁴ C]lysine		Glycosylated hydroxy[¹⁴ C]lysine	
	(10 ⁻⁴ × d.p.m.)	Change (%)	(10 ⁻³ × d.p.m.)	Change (%)	(10 ⁻⁴ × d.p.m.)	Change (%)	(10 ⁻³ × d.p.m.)	Change (%)	(10 ⁻² × d.p.m.)	Change (%)
Control	9.8		25.5		9.54		10.10		27.50	
+Cortisol	6.8	-31	7.3	-71	8.28	-13	2.26	-78	8.30	-70

Table 3. *Effect of cortisol acetate on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase in whole chick embryos*

The experimental conditions were the same as in Table 2. Supernatant protein (10–100 µg) was used in the enzyme assays. The values are expressed as $10^{-4} \times \text{d.p.m.}$ per mg of the 15000g-supernatant protein and are the means of duplicate or triplicate samples from one experiment.

Cells	Prolyl hydroxylase activity		Lysyl hydroxylase activity		Galactosyltransferase activity		Glucosyltransferase activity	
	($10^{-4} \times \text{d.p.m.}$)	Change (%)	($10^{-4} \times \text{d.p.m.}$)	Change (%)	($10^{-4} \times \text{d.p.m.}$)	Change (%)	($10^{-4} \times \text{d.p.m.}$)	Change (%)
Control	1.27		9.60		0.41		0.79	
+Cortisol	0.80	-37	6.20	-35	0.30	-27	0.58	-27

and the tendon cells were isolated after incubation for 24 h. When the cells were pulsed with [^{14}C]proline there was a marked decrease in total protein synthesis (Table 1), that noted in the formation of hydroxy[^{14}C]proline being considerably larger than that in the other measure of protein synthesis. Prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities were assayed as described in the Experimental section. To ensure that the assays of all the enzyme activities were carried out under conditions in which the relationship between enzyme activity and product formation was linear, standard curves were prepared for all enzymes. Usually 10–20 µg of supernatant protein from the cell homogenates or 10–100 µg from the whole embryos was used in the enzyme assays. In this experiment there was no change in prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities (results not shown).

In the next experiments, cortisol acetate was injected four times (100 µg/embryo daily) into fertilized eggs, and the tendon cells and the whole embryos were analysed 24 h after the last injection. Some of the cells were incubated with [^{14}C]proline or [^{14}C]lysine and the rest were used for the assay of the enzyme activities and immunoreactive prolyl hydroxylase protein. The mean weight of the embryos was 16 g in the controls and 12.4 g in the cortisol-treated group. Incorporation of [^{14}C]proline decreased by about 30 % and the synthesis of hydroxy-[^{14}C]proline by about 70 % (Table 2). The incorporation of [^{14}C]lysine decreased only by about 13 %, but the synthesis of hydroxy[^{14}C]lysine and glycosylated hydroxy[^{14}C]lysine decreased by about 70–78 %.

Since the ratio of radioactivity in hydroxylysine to total radioactivity may be affected either by the degree of hydroxylation of the lysine residues in collagen or by the amount of collagen synthesis relative to total protein synthesis, an attempt was made to estimate the degree of hydroxylation of the lysine residues in the collagen itself. Collagen syn-

thesized by the chick-embryo tendons, after similar treatment with cortisol acetate as described above, was digested with 0.5 mg of purified bacterial collagenase (Sigma) for 24 h at 37°C in 0.02 M- NH_4HCO_3 /0.1 mM- CaCl_2 , pH 7.5. Diffusible peptides were then assayed for total radioactivity in free hydroxylysine and substituted hydroxylysine. No essential changes were found in the ratio of total hydroxylysine to lysine, though that of glycosylated hydroxylysine to total hydroxylysine was 0.30 in the tendons of controls and 0.35 in those of cortisol-treated embryos.

Effect of cortisol acetate administration on enzyme activities in the whole embryos and the isolated tendon cells

A decrease was found in the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase in the whole embryos after four injections of cortisol acetate (100 µg/embryo daily) (Table 3), that in the two hydroxylase activities being larger than in the two transferase activities. The enzyme activities and the amount of immunoreactive prolyl hydroxylase protein were also determined in the isolated tendon cells (Table 4). All these enzyme activities decreased, the decrease in the hydroxylase activities again being slightly larger than that in the transferase activities. The amount of immunoreactive prolyl hydroxylase protein diminished to the same extent as the prolyl hydroxylase activity, and thus there was no change in the specific activity of prolyl hydroxylase when calculated per unit of enzyme protein.

Reversal of the inhibitory effect of cortisol acetate

Cortisol acetate was injected twice on to the chorioallantoic membrane of chick embryos, and the tendon cells were isolated 3 days after the last injection. The cells were then incubated with [^{14}C]proline or [^{14}C]lysine as described in the Experimental section. The incorporation of [^{14}C]proline or [^{14}C]lysine was the same in the cortisol-treated cells as in the control cells (Table 5), although

Table 4. *Effect of cortisol acetate on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase in tendon cells*
The experimental conditions were the same as in Table 2. Enzyme activities were calculated per 10⁶ tendon cells. Two separate portions of the cells (I and II) were homogenized and assayed in duplicate or triplicate; 10–20 µg of protein was used in the enzyme assays. The results are means of duplicate or triplicate samples in one experiment.

Cells	Prolyl hydroxylase activity		Lysyl hydroxylase activity		Galactosyltransferase activity		Glucosyltransferase activity		Immunoreactive prolyl hydroxylase	
	(10 ⁻³ × d.p.m.)	Change (%)	(10 ⁻³ × d.p.m.)	Change (%)	(10 ⁻² × d.p.m.)	Change (%)	(10 ⁻² × d.p.m.)	Change (%)	(µg)	Change (%)
Control I	14.20		8.50		6.79		18.7		1.47	
II	12.50		7.70		7.15		15.6		—	
+Cortisol I	5.95		4.05		3.92		9.3		0.65	
II	7.15	-51*	4.15	-49*	4.91	-37*	10.9	-41*	—	-56

* Change (%) has been calculated from the mean values of the homogenates I and II.

the synthesis of hydroxy[¹⁴C]proline and hydroxy-[¹⁴C]lysine was decreased by about 20% in the cells of the steroid-treated embryos. Prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities were assayed in the cells as described in the Experimental section. All four enzyme activities were lower in the cells of the steroid-treated embryos (Table 6), though the glycosyltransferase activities were decreased less than the hydroxylase activities.

Effect of cortisol acetate on collagen synthesis and enzyme activities in vitro

The effect of cortisol acetate on collagen synthesis was studied further by adding the steroid directly to the incubation medium. With a cortisol acetate dose of 1 mg/ml there was a decrease of about 40% both in the total protein synthesis and in the formation of hydroxyproline (results not shown).

To study the effect of cortisol acetate on the enzyme activities, the steroid was added directly to the reaction mixture with each enzyme separately. The enzymes were prepared as described in the Experimental section. A dose of 100 µg of cortisol acetate/ml had no effect on the enzyme activities (results not shown).

Discussion

The activity of prolyl hydroxylase in tissues increases in a number of conditions accompanied by an increased rate of collagen biosynthesis and is low in conditions associated with a slow rate of collagen synthesis (see Kivirikko & Risteli, 1976). The other three enzyme activities catalysing intracellular post-translational modifications in biosynthesis of collagen have been studied only in a few conditions (see Kivirikko & Risteli, 1976), but work that has been carried out suggests that these enzyme activities also increase in conditions accompanied by an increased rate of collagen biosynthesis. However, the four enzyme activities do not change in an entirely similar manner, and larger changes are found in the prolyl hydroxylase and lysyl hydroxylase activities than in the two collagen glycosyltransferase activities in rat liver (Risteli & Kivirikko, 1976) and human skin (Anttinen *et al.*, 1977) with age, and in rat liver in hepatic injury (Risteli & Kivirikko, 1974, 1976). This suggests that the enzyme activities may be regulated individually.

In the present study the injection of cortisol acetate produced changes in enzyme activities and collagen synthesis, but there was a certain time lag before the changes in the enzyme activities occurred. When the steroid was injected 1 day before the isolation of the cells, the enzyme activities were similar to those in the control cells, even though

Table 5. *Reversal of cortisol acetate inhibition of the incorporation of [14 C]proline and [14 C]lysine and the formation of hydroxy[14 C]proline and hydroxy[14 C]lysine in tendon cells*

Two injections of cortisol acetate (200 μ g/embryo) were given at an interval of 24 h, and the tendon cells were isolated 3 days after the last injection, when the embryos were 17 days old. The tendon cells (10×10^6) were incubated with 2 μ Ci of [14 C]proline or 1 μ Ci of [14 C]lysine in 2 ml of a modified Krebs medium for 2 h and total radioactivity, hydroxy[14 C]proline and hydroxy[14 C]lysine were assayed as described in the Experimental section. The values are the means of duplicate samples from one experiment. Total [14 C]proline is the sum of [14 C]proline and hydroxy[14 C]proline and total [14 C]lysine the sum of [14 C]lysine and hydroxy[14 C]lysine.

Cells	Total [14 C]proline		Total hydroxy[14 C]proline		Total [14 C]lysine		Total hydroxy[14 C]lysine	
	($10^{-4} \times$ d.p.m.)	Change (%)	($10^{-4} \times$ d.p.m.)	Change (%)	($10^{-4} \times$ d.p.m.)	Change (%)	($10^{-4} \times$ d.p.m.)	Change (%)
Control	34.0		11.2		7.5		0.80	
+Cortisol	34.2	0	9.3	-17	7.5	0	0.65	-19

Table 6. *Reversal of the effect of cortisol acetate on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase in tendon cells*

The experimental conditions were the same as in Table 5. Enzyme activities were assayed as described in the Experimental section. Enzyme activities are expressed per mg of protein of the 15000g supernatant. The values are the means of duplicate samples.

Cells	Prolyl hydroxylase activity		Lysyl hydroxylase activity		Galactosyltransferase activity		Glucosyltransferase activity	
	($10^{-3} \times$ d.p.m.)	Change (%)	($10^{-3} \times$ d.p.m.)	Change (%)	($10^{-3} \times$ d.p.m.)	Change (%)	($10^{-3} \times$ d.p.m.)	Change (%)
Control	95.2		13.0		10.7		26.0	
+Cortisol	60.7	-36	9.0	-31	9.5	-11	20.0	-23

protein synthesis and collagen synthesis had been markedly decreased by this stage, and the rate of collagen synthesis was lower than that of other proteins. Repeated injections of cortisol acetate also produced marked decreases in the enzyme activities, and these changes were found regardless of whether the activities were assayed in whole chick embryos or directly in tendon cells. Interestingly, the changes in the two transferase activities were smaller than those in the two hydroxylase activities, whereas in rat liver a similar decrease occurred in all four enzyme activities after treatment with prednisolone (J. Risteli, personal communication).

Since the enzyme activities were assayed here directly in the cells synthesizing the collagen, changes in number of cells do not produce any effect on these results.

Prolyl hydroxylase is present in tissues in two forms, as an active tetramer and as monomers (McGee & Udenfriend, 1972; Stassen *et al.*, 1973, 1974). The relationship between the active form and the monomers may change in relation to the activity of collagen synthesis (Stassen *et al.*, 1974; Kao *et al.*, 1975; J. Risteli *et al.*, 1976; Tuderman, 1976; Tuderman & Kivirikko, 1977). Even though these two forms were not directly studied here by gel filtration, it is possible to calculate from the changes

in the enzyme activity and the immunoreactive prolyl hydroxylase protein that the ratio of the tetramer to the monomers was the same in the control cells and in the cells treated with cortisol acetate. This indicates that the steroid does not affect the process of prolyl hydroxylase activation, but does affect the rate of synthesis of the enzyme protein. This is consistent with findings indicating that anti-inflammatory steroids have no effect on the activity of prolyl hydroxylase in cultured fibroblasts (Cutroneo *et al.*, 1975) or in isolated cells *in vitro* (Oikarinen, 1977). In the present study, cortisol acetate similarly had no effect on the enzyme activities *in vitro*.

The changes in enzyme activities induced by cortisol acetate were partly reversible (see also Cutroneo *et al.*, 1975). Because the period of drug deprivation was only 3 days, it may be that a longer period could have restored the enzyme activities completely. Interestingly, total protein synthesis was restored completely, but collagen synthesis only partially. This inhibition in collagen synthesis may be due to the decrease in the enzyme activities.

Since changes in the enzyme activities may produce changes in the extent of the post-translational modifications, hydroxylysine and glycosylated hydroxylysine were determined after collagenase digestion. There was no change in the ratio of hydroxylysine to lysine in the collagen synthesized

after treatment with cortisol acetate, although lysyl hydroxylase activity was decreased. It may be noted that the rate of collagen synthesis was also decreased, as indicated by the incorporation experiments, and it thus seems possible that there was enough enzyme activity to catalyse the hydroxylation normally under these conditions. There was, however, a slight increase in the ratio of glycosylated hydroxylysine to total hydroxylysine, indicating that the collagen synthesized may contain slightly more glycosylated hydroxylysine.

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