INHIBITION OF PROLINE AND LYSINE HYDROXYLATION PREVENTS NORMAL EXTRUSION OF COLLAGEN BY 3T6 FIBROBLASTS IN CULTURE

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1. Introduction

Although hydroxyproline occurs universally in all vertebrate collagens [1], its function is unknown. Autoradiographic studies of embryonic chick tibiae incubated in vitro indicated that collagen molecules having a decreased content of *trans*-hydroxyproline [2] or hydroxylysine [3] or both [4–7] were not extruded at a normal rate and accumulated intracellularly. Recently Gribble et al. [8] have reported that rapidly dividing L-929 fibroblasts extrude collagenous molecules with a reduced hydroxyproline content. In order to clarify the relationship between the hydroxylation process and the extrusion of collagen we have grown the mouse fibroblast line 3T6 under conditions in which collagen is synthesized and extruded in a soluble form into the medium above the cell layer. When the ^{14}C-proline or ^{14}C-lysine labeled proteins in the medium were chromatographed on Bio-Gel A-1.5, most of the label in collagenous proteins appeared in a peak corresponding to a molecular weight somewhat larger than β chains (200,000 daltons). By varying the concentration of the Fe^{2+} chelator, α,α'-dipyridyl, in the incubation medium, partial to complete inhibition of proline and lysine hydroxylation was achieved. As the inhibition increased, there was a progressive decrease in the quantity of radioactivity of collagenous protein in the high molecular weight peak with complete disappearance at maximal inhibition.

2. Materials and methods

The mouse fibroblast line 3T6 obtained from Dr. Howard Green [9] was maintained in Dulbecco-Vogt modified Eagles medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum and 50 μg/ml sodium ascorbate in an atmosphere of 10% CO_2 –90% air. The cells were passaged every second or third day. Perforated cellophane (Microbiological Associates) was routinely used to detach the cells from the culture flasks, and they were resuspended with a pipet. Further details of incorporation experiments and analytical procedures are given in the tables and figures.

Bacterial collagenase from Worthington Biochemical Corp. was further purified by the method of Harper and Kang [11]. Radiochemicals and other materials were obtained as previously described [5]. The ^{14}C-hydroxyproline content of samples labeled with ^{14}C-proline was assayed by a specific chemical procedure [12], and the ^{14}C-hydroxylysine content of samples labeled with ^{14}C-lysine by chromatography

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Table 1
Incorporation of ^{14}C-proline into proteins in the cell layer and medium and ^{14}C-hydroxyproline content of these proteins.

Time incubation with ^{14}C-proline	Cells		Medium	
	Total ^{14}C	Degree of hydroxylation[a]	Total ^{14}C	Degree of hydroxylation[a]
(hr)	(dpm)	(%)	(dpm)	(%)
Control				
2	97,639	0.6	4,144	12.8
4	207,903	0.65	13,982	17.0
6	308,004	1.0	24,466	19.3
8	383,376	0.7	36,153	18.2
α,α'-Dipyridyl (10^{-4} M)				
2	92,141	< 0.3	3,380	< 0.3
4	163,018	< 0.3	11,258	< 0.3
6	196,084	< 0.3	16,752	< 0.3
8	195,505	< 0.3	16,229	< 0.3

7×10^5 cells in 2.5 ml were plated onto 50 mm petri dishes and incubated for 60 hr. The medium was then replaced with fresh medium containing 1 μCi ^{14}C-proline and the incubation continued for the periods indicated. At the end of the incubation, medium and cells were dialyzed separately and the proteins analyzed for total ^{14}C incorporation and ^{14}C-hydroxyproline content as described in the text. In the experiments with 10^{-4} M α,α'-dipyridyl, the α,α'-dipyridyl was added 20 min before the isotope. The values represent the average of replicate platings.
[a] Values are 100 times ^{14}C-hydroxyproline per total ^{14}C.

on the short column of a Beckman amino acid analyzer [13].

3. Results

Cells incubated with ^{14}C-proline for 2–8 hr incorporated isotope continuously into the proteins of both the cell layer and medium (table 1, control). Although the majority of the counts were in the cells, most of the hydroxyproline was found in the medium. The fraction of the counts in the medium which were in ^{14}C-hydroxyproline (degree of hydroxylation) increased somewhat from 13% after 2 hr of incubation to approximately 20% after 6 to 8 hr.

In order to estimate the molecular weight of the proteins in the medium, the medium from cells incubated for 8 hr with either ^{14}C-proline or ^{14}C-lysine was placed onto the Bio-Gel column (fig. 1). The distribution of counts was similar with both isotopes and for further study the counts were grouped into two fractions as indicated in fig. 1. After dialysis of the pooled fractions to remove $CaCl_2$, they were incubated with purified collagenase and the digest mixture then dialyzed against distilled water. The peptides released by collagenase and the retained peptides were then analyzed for either ^{14}C-hydroxyproline or ^{14}C-hydroxylysine content (table 2, control). When the proteins were labeled with ^{14}C-proline, greater than 80% of the total from region A was released by collagenase, and the degree of hydroxylation of these peptides, 41% was close to that expected for pure collagen [1]. Only about 14% of the total ^{14}C of region B was released by collagenase. When the proteins were labeled with ^{14}C-lysine, approximately 69% of the total ^{14}C in region A was released by collagenase, and the degree of hydroxylation was 42%. Basic hydrolysis [13] of aliquots of these peptides indicated that about 40% of the hydroxylysine residues were glycosylated.

In order to determine the concentrations of α,α'-dipyridyl necessary for partial and maximal inhibition of hydroxylation, cells were incubated with ^{14}C-proline and varying concentrations of α,α'-dipyridyl for 8 hr (table 3). A concentration of 10^{-4} M produced maximal inhibition while 10^{-5} M was completely ineffective. At 10^{-4} M α,α'-dipyr-

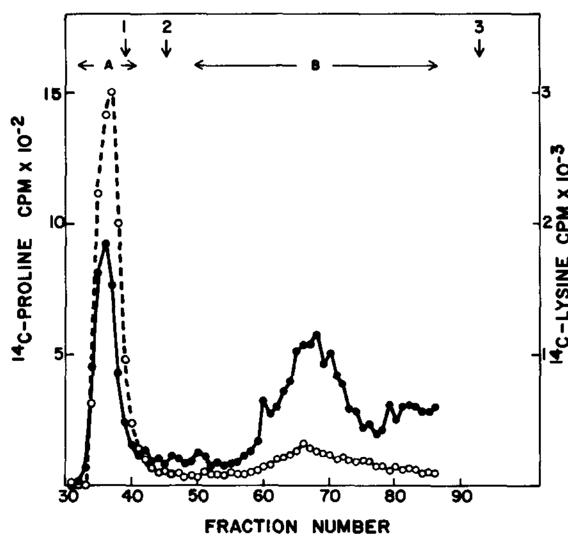

Fig. 1. Gel chromatography of soluble ^{14}C-labeled proteins in the medium. Cells were incubated with either 20μCi of ^{14}C-proline or 30 μCi of ^{14}C-lysine for 8 hr. The medium above the cell layer was removed and placed on a 1.9 × 114 cm Bio-Gel A-1.5, 200–400 mesh column, equilibrated with 1 M $CaCl_2$, 0.05 M tris pH 7.6 [10]. The flow rate was maintained at 15 ml/hr by a peristaltic pump, and 3.75 ml fractions were collected. Two tenths ml aliquots of these were counted in a scintillation counter. The fractions were pooled into the two indicated regions. A and B, for further study. ^{14}C-proline (o- - -o); ^{14}C-lysine (•——•). Number 1 indicates the chromatographic position of β chains, 2 the position of α chains, and 3 the included volume of the column.

idyl only a minimal effect on total incorporation was seen during 2 to 4 hr incubations, but a 50% inhibition was seen in 8 hr incubations (table 1). This decrease may reflect control of protein synthesis and is currently under investigation.

Since significant amounts of ^{14}C-proline continued to appear in the medium at all concentration of α,α'-dipyridyl, samples of the medium were chromatographed on the Bio-Gel column. Fig. 2 demonstrates that as the concentration of α,α'-dipyridyl was increased, there was a progressive diminution in the quantity of radioactivity in region A and at 10^{-4} M α,α'-dipyridyl the peak was virtually absent and all the radioactivity was in region B. Collagenase digestion of these peaks revealed that as the concentration of α,α'-dipyridyl increased, the fraction of the total ^{14}C in region A which was released by the collagenase decreased until none was released at 10^{-4} M α,α'-dipyridyl. In contrast, there was an increase in the fraction of counts of region B which were released by collagenase when there was significant inhibition of hydroxylation (table 2).

In order to show that unhydroxylated molecules accumulated intracellularly two types of experiments were performed (table 4). In the first experiment, cells were incubated with ^{14}C-proline with or without 10^{-4} M α,α'-dipyridyl and then the label was chased with fresh medium containing ^{12}C-proline and 10^{-3} M

Table 2
Collagenase digestion of ^{14}C-labeled proteins obtained by gel chromatography of medium after incubating cells with varying concentrations of α,α'-dipyridyl.

α,α'-Dipyridyl during labeling of cells	Peptides released by collagenase treatment				
	Region A		Region B		A total ^{14}C / B total ^{14}C
(M)	Fraction of total ^{14}C (%)	Degree of hydroxylation[a] (%)	Fraction of total ^{14}C (%)	Degree of hydroxylation[a] (%)	
0[b]	69	42.4	–	–	0.34
0[c]	84	41.0	14	–	1.6
5×10^{-5} [c]	58	38.5	24	3.1	0.26
10^{-4} [c]	< 1	–	23.8	–	< 0.05

Pooled fractions from region A and B were dialyzed exhaustively against distilled water to remove $CaCl_2$ and then were digested with 50 μg/ml purified collagenase in 50 mM tris-HCl pH 7.2, 5 mM $CaCl_2$ for 24 hr at 37°. The enzyme digest mixture was dialyzed against 5 volumes of distilled water and the dialysate and retentate were hydrolyzed and analyzed for ^{14}C-hydroxyproline or ^{14}C-hydroxylysine.

[a] Values are 100 times ^{14}C-hydroxyproline per total ^{14}C in tissues labeled with ^{14}C-proline and 100 times ^{14}C-hydroxylysine plus ^{14}C-lysine in tissues labeled with ^{14}C-lysine.

[b] Tissues labeled with ^{14}C-lysine.

[c] Tissues labeled with ^{14}C-proline.

Table 3
Effect of varying concentration of α,α'-dipyridyl on the formation of ^{14}C-hydroxyproline.

α,α'-Dipyridyl	Cells		Medium	
(M)	Total incorporation (dpm)	Degree of hydroxylation[a] (%)	Total incorporation (dpm)	Degree of hydroxylation[a] (%)
0	548,948	1.4	130,224	18.2
10^{-5}	570,450	1.0	120,384	23.0
5×10^{-5}	515,304	0.3	33,320	12.0
7.5×10^{-5}	382,553	< 0.3	24,257	3.1
10^{-4}	222,757	< 0.3	22,033	< 0.3

7×10^5 cells were plated onto a 50 mm petri dish and incubated for 60 hr. The medium was then replaced with fresh medium containing the indicated concentration of α,α'-dipyridyl. Twenty minutes later 2 μCi of ^{14}C-proline was added and the incubation continued for 8 hr. The medium and the cells were then separated, dialyzed free of unincorporated ^{14}C-proline and the retained radioactivity analyzed for ^{14}C-hydroxyproline. Values represent the average of duplicate platings.
[a] Values are 100 times ^{14}C-hydroxyproline per total ^{14}C.

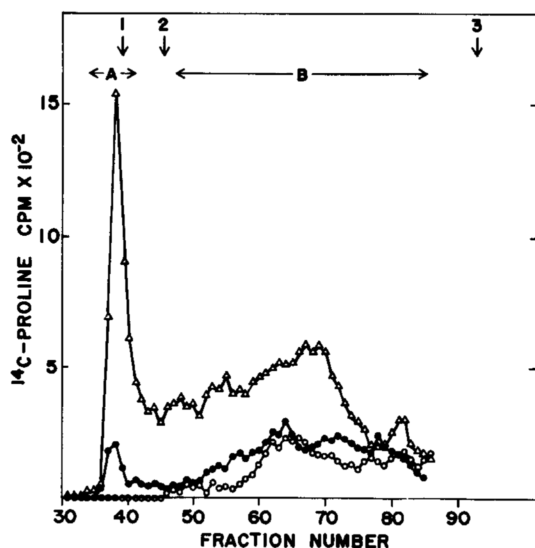

Fig. 2. Effect of varying concentration of α,α'-dipyridyl on the gel chromatographic pattern of ^{14}C-labeled proteins in the medium. Cells were incubated with 30 μCi of ^{14}C-proline for 8 hr and the medium was then collected and chromatographed as described in fig. 1 except that 0.4 ml aliquots were counted. 5×10^{-5} M α,α'-dipyridyl (△——△); 7.5×10^{-5} M (●——●); 10^{-4} M (○——○). Number 1 indicates the chromatographic position of β chains, 2 the position of α chains, and 3 the included volume of the column.

$FeSO_4$ to reverse the inhibition of hydroxylation. The results demonstrated that approximately twice as much ^{14}C-hydroxyproline was extruded into the medium during the chase period by the cells treated with α,α'-dipyridyl as by the control cells. In the second experiment, cells were incubated with ^{14}C-proline with or without 10^{-4} M α,α'-dipyridyl for 3 hr and then the soluble proteins from the homogenized cell layers were used as substrate for protocollagen proline hydroxylase [5]. The results demonstrated that approximately 4 times as much hydroxyproline was formed with the substrate prepared from the α,α'-dipyridyl treated cells as with that from the control cells.

4. Discussion

Although hydroxyproline and hydroxylysine occur in all vertebrate interstitial collagens, they do not seem to be necessary for the structural integrity of the collagen molecule, and their function remains obscure. Previous experiments with embryonic tibiae indicated that when the hydroxylation of proline and lysine was inhibited, unhydroxylated molecules were extruded at a markedly reduced rate if at all [4]. When tibiae were incubated with analogues of proline [2, 5–7] or lysine [3], collagen molecules containing significantly decreased amounts of either *trans*-4-hydroxyproline or hydroxylysine were syn-

Table 4
Evidence for the intracellular accumulation of unhydroxylated molecules.

Sample	Medium		Cells	
	Total dpm	Degree of hydroxylation[a] (%)	Total dpm	Degree of hydroxylation[a] (%)
Expt. 1				
Before chase				
(a) Control	37,109	23.9		
(b) 10^{-4} M α,α'-dipyridyl	24,445	< 1		
After chase				
(a) Control	15,526	20.6	412,595	< 0.4
(b) 10^{-4} M α,α'-dipyridyl	21,142	30.0	324,429	0.5
Expt. 2				
Before hydroxylation				
(a) Control			225,000	0.6
(b) 10^{-4} M α,α'-dipyridyl			203,000	< 0.3
After hydroxylation				
(a) Control			225,000	2.1
(b) 10^{-4} M α,α'-dipyridyl			203,000	8.1

7×10^5 cells were plated onto each 50 mm dish and incubated for 60 hr. The medium was then replaced with fresh medium with or without 10^{-4} M α,α'-dipyridyl. Twenty minutes later ^{14}C-proline was added and the incubation continued for 3 hr. In experiment 1 the medium was then replaced with medium containing 25 μg/ml ^{12}C-proline and 10^{-3} M $FeSO_4$, and the incubation continued for another 3 hr. The two sets of media and the cell layer were then analyzed as described in table 1. In experiment 2, the cells were collected and homogenized in 0.05 M tris, pH 7.6, 0.2 M KCl and then dialyzed exhaustively against the same buffer. The dialyzed homogenate was centrifuged at 100,000 *g* for 1 hr and aliquots of the supernatant used as substrate with excess protocollagen proline hydroxylase to achieve maximal hydroxylation [5].
[a] Values are 100 times ^{14}C-hydroxyproline per total ^{14}C.

thesized. These molecules also seemed to be extruded much more slowly than normally. These experiments suggested that both *trans*-4-hydroxyproline and hydroxylysine were each necessary but not sufficient requirements for normal extrusion. However, qualitative autoradiographic techniques were used to determine extrusion and it was not possible to quantitate or determine the nature of the proteins which continued to be extruded in this system. Recently Gribble et al. [8] have reported that rapidly dividing L-929 mouse fibroblasts extrude collagenous molecules with a significantly decreased degree of hydroxylation.

The present work demonstrates that 3T6 fibroblasts in late logarithmic-early stationary phase growth extrude most of the synthesized collagen into the medium above the cell layer. This collagen when chromatographed on Bio-Gel A-1.5 under denaturing conditions appeared in a peak corresponding to a molecular weight somewhat greater than 200,000 daltons. Little material was found in the position of α chains, but significant amounts in the position of 10,000–50,000 molecular weight peptides. Layman et al. [14] have found that the medium taken from confluent layers of human fibroblasts contains collagen which does not yield α chains under denaturing conditions. They have suggested that this might represent a transport form of collagen, and the collagen in the present system may function in a similar capacity.

It was possible in the present system to vary the inhibition of proline hydroxylation by using graded concentrations of α,α'-dipyridyl. As the degree of inhibition increased, the quantity of collagen in the

large molecular weight fraction (region A) decreased progressively. However, the degree of hydroxylation of the collagen which remained in this region (obtained by digestion with collagenase) remained high. When the inhibition of hydroxylation was maximal, no collagenous protein was found in region A. In contrast, the quantity of radioactivity found in region B of the chromatograms and which corresponded to molecular weights no greater than one-half that of single α chains remained high at all α,α'-dipyridyl concentrations. The fraction of radioactivity of region B which was released by collagenase also increased when the hydroxylation was significantly inhibited. The unhydroxylated molecules (protocollagen) accumulated intracellularly and were extruded when the inhibition of hydroxylation was reversed. The protocollagen could be isolated from the cell layer and served as substrate for purified protocollagen proline hydroxylase (table 4). These experiments strongly suggest that inhibition of hydroxylation prevents normal extrusion from the cell and that some of these retained molecules are then partially degraded and the fragments extruded.

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