METABOLISM BY ISOLATED FIBROBLASTS OF ABNORMAL COLLAGENS CONTAINING ANALOGUES OF PROLINE OR LYSINE

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1. Introduction

Although hydroxyproline and hydroxylysine occur in all vertebrate interstitial collagen [1], 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 and isolated fibroblasts [2–4] indicated that when the hydroxylation of proline and lysine was inhibited, unhydroxylated molecules were extruded at a markedly reduced rate. In the case of the 3T6 fibroblasts in culture, the retained molecules appeared to be slowly degraded intracellularly. When tibiae were incubated with analogues of proline [5–8] or lysine [9], collagen molecules containing significantly decreased amounts of either *trans*-hydroxyproline or hydroxylysine or both were synthesized. These molecules also seemed to be extruded much more slowly than normally, and these experiments suggested that both *trans*-hydroxyproline and hydroxylysine are each necessary but not sufficient requirements for normal extrusion. The present results using fibroblasts isolated from embryonic chick tendons and incubated in suspension confirm and extend the previous finding that collagen containing proline or lysine analogues are retained intracellularly. Whereas normal collagen molecules in this system are secreted into the surrounding incubation medium within 30 min after they are synthesized, molecules containing either dehydroproline* or *cis*-hydroxyproline* are retained for a minimum of 150 min and are probably degraded intracellularly. The results are less definitive when lysine is replaced by dehydrolysine*, although it is clear that these abnormal molecules are also secreted much more slowly than normal. In addition, continued incubation of the fibroblasts with the analogues leads to a significant reduction in the rate at which collagen is synthesized.

* DL-3, 4-dehydroproline is referred to as dehydroproline; *cis*-hydroxy-L-proline as *cis*-hydroxyproline; and DL-4, 5-*trans*-dehydrolysine as dehydrolysine.

2. Materials and methods

Fibroblasts were isolated from 17 day old chick embryo tendons as described by Dehm and Prockop [4] except that the digestion with bacterial collagenase and trypsin was performed in Eagle's minimum essential medium containing 2% fetal calf serum. The morphologic appearance of the cells when examined in the electron microscope was better preserved when the serum was added. The subsequent incubations at 4×10^6 cells/ml were performed in Krebs medium with 2% serum, 50 μg/ml ascorbic acid, radioisotope and amino acid analogues. One ml aliquots were taken, chilled and centrifuged at 1500 *g* for 10 min to separate the cells from the medium. The medium and cell fractions were either dialyzed against running tap water and the dialysates hydrolyzed and assayed by a specific procedure for their [^{14}C] hydroxyproline content [10] or the fractions were prepared for disc gel electrophoresis (fig. 3). Disc gel electrophoresis in sodium dodecyl sulfate (SDS) was performed as described by Weber and Osborn [11] using 10% acrylamide gels and half the standard amount of cross-linker. The gels were cut into 1.8 mm fractions which were solubilized and counted in a scintillation

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counter [12]. Dehydrolysine was synthesized as previously described [9], and *cis*-hydroxyproline and dehydroproline were purchased from Calbiochem. Radiochemicals and other materials were obtained as previously described [3].

3. Results

Based upon previous experience with embryonic chick tibiae and upon preliminary experiments with these cells the following concentrations of analogues were chosen for more detailed investigation: dehydroproline, 100 μg/ml; *cis*-hydroxyproline, 400 μg/ml; dehydrolysine, 800 μg/ml. It is believed that these concentrations produce a high degree of replacement by the analogue for the normal amino acid in the synthesized proteins without producing significant non-specific effects [7–9].

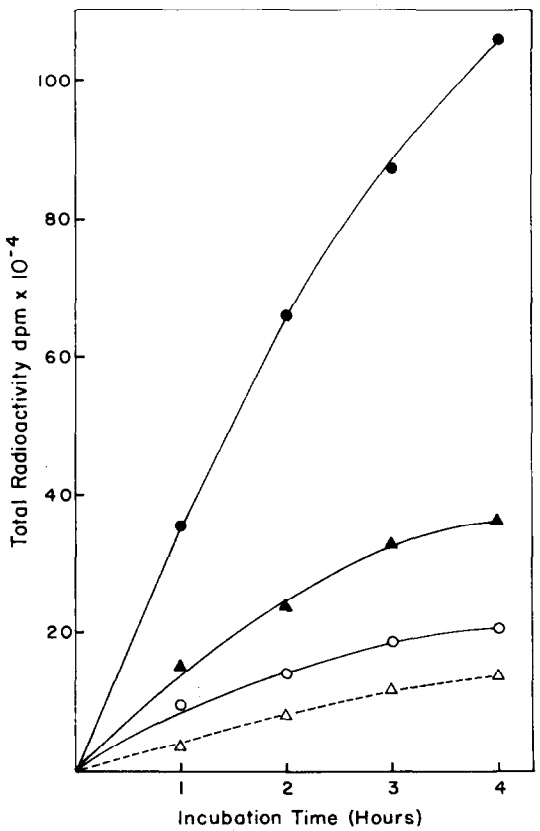


Fig. 1. Incorporation of [^{14}C] proline into protein by isolated cells in the presence and absence of analogues. Cells were incubated with 1 μCi/ml (specific activity 100 μCi/μmole) [^{14}C] proline and 1 ml aliquots taken at the indicated times. Values indicate non-dialyzable ^{14}C in the total system (cells plus medium). Control (●-●-●); dehydroxyproline, 100 μg/ml (Δ-Δ-Δ); *cis*-hydroxyproline, 400 μg/ml (o-o-o); dehydrolysine, 800 μg/ml (▲-▲-▲).

When cells were incubated with [^{14}C] proline in the absence of any added analogue, incorporation continued at a linear rate during the first 2 hr and then declined so that the incorporation rate during the final hour was approx. 50% of that in the first hour. Notice that after 4 hr of incubation about 50% of the available isotope had been incorporated into protein (fig. 1). When either dehydroproline or *cis*-hydroxyproline were included in the incubation medium there was a marked inhibition of incorporation due to substitution by the analogues for [^{14}C] proline. In addition the decline in the incorporation rate during the fourth hour was proportionately much greater than occurred in the control even though less than 10% of the available [^{14}C] proline had been incorporated in the presence of the analogues. Qualitatively similar results were seen with dehydrolysine except there was much less inhibition of incorporation at all times. The fraction of the incorporated label which was secreted into the medium increased from 46.0% after 1 hr of incubation to 76.6% after 4 hr of incubation in the control cells (fig. 2 and table 1). There was a marked reduction in the rate at which label appeared in the medium when any one of the three analogues was included during the incubation. Results of the [^{14}C] hydroxyproline determinations on proteins synthesized in the presence of analogues were consistent with previous findings in the chick tibiae. There was a pronounced inhibition of hydroxylation with dehydroproline and little effect with dehydrolysine. The inhibition of hydroxylation seen here with *cis*-hydroxyproline was slightly greater than that seen in the tibiae at the same analogue concentration.

Because of the marked inhibition of [^{14}C] proline incorporation by the analogues further experiments were performed using [^{3}H] glycine. The main purposes of these experiments were to i) compare the molecular weights of the proteins being synthesized and secreted by the cells in the presence and absence of the analogues, and ii) determine whether the retained molecules were degraded. Cells were labeled with [^{3}H] glycine for 30 min and then they were incubated with excess unlabelled glycine for up to 150 min. The proteins found in the medium and cells

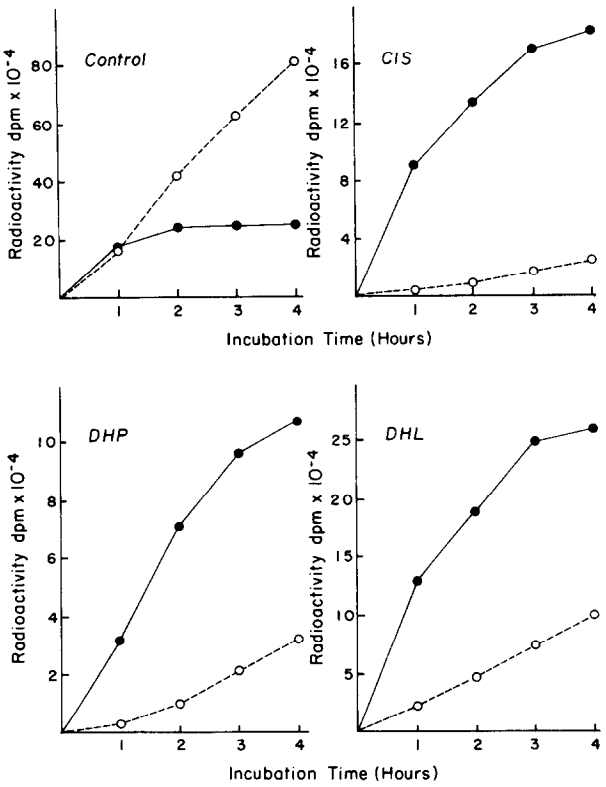


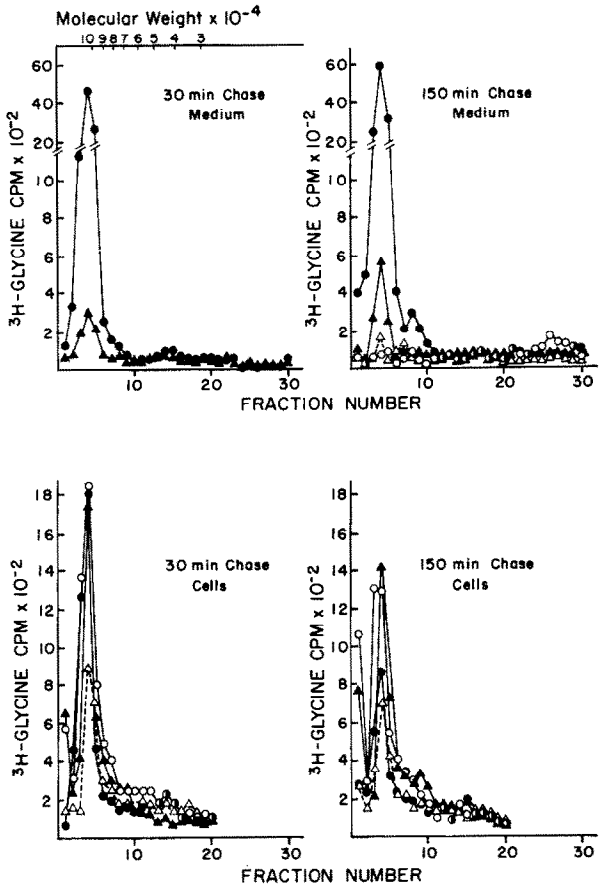
Fig. 2. Incorporation of [^{14}C] proline into cells and medium. Values indicate incorporation into cells (●-●-●) and medium (o-o-o) from the experiment described in fig. 1.

were solubilized using 1% SDS and 1% mercaptoethanol (fig.3 and table 2) and electrophoresed on polyacrylamide gels. We have noted some irregularity in the ability to completely stop [^{3}H] glycine incorporation (see control samples, table 2) during the chase period but nevertheless the results are clear cut in spite of this difficulty. In the control cells after a chase period of 150 min, 75% of the incorporated [^{3}H] glycine was found in the medium. Much smaller fractions were found in the medium when any one of the analogues were included. In the control cells approx. 85% of the radioactivity in the medium was found in a peak corresponding to a molecular weight somewhat greater than 100,000 daltons and which appears therefore to be a form of procollagen (fig. 3) [13, 14]. With either of the proline analogues less than 10% of the label in the medium was found in the position corresponding to the procollagen and most was in smaller molecular weight peptides. However, in the case of dehydrolysine about 55% of the label in the medium was found in a position identical to the control. The distribution of radioactivity in the proteins in the cells was more heterogeneous but a well defined peak identical in position to that found in the medium was observed in all samples. As seen in fig. 3 there was a decrease of about 20% in the amount of label found in this intracellular peak during the chase in-

Table 1
Distribution of total radioactivity between cells and medium, and hydroxylation of [^{14}C] proline in the presence and absence of analogues.

Sample incubation time (hr)	Cells, degree of hydroxylation* (%)	Medium, degree of hydroxylation* (%)	Total system, degree of hydroxylation* (%)	$\frac{\text{Medium cpm}}{\text{Total cpm}} \times 100$ (%)
Control				
1	28.1	40.4	34.1	48.6
4	12.9	39.0	32.9	76.6
cis-Hypro				
1	24.4	10.5	23.9	4.3
4	22.2	12.7	21.1	11.9
Dehydropro				
1	9.0	8.8	9.0	8.6
4	7.8	11.3	8.6	22.9
Dehydrolysine				
1	32.6	37.1	33.3	14.8
4	31.3	28.0	30.8	27.9

*Values are 100 X [^{14}C] hydroxyproline per total ^{14}C.
Degree of hydroxylation was determined on cell and medium fractions of experimental values given in figs. 1 and 2.



terval of 30 min to 150 min in the cells incubated with analogues.

4. Discussion

Previous experiments using qualitative autoradiographic techniques suggested that collagen molecules

Fig. 3. Gel electrophoresis of [^{3}H] glycine labeled proteins in the cells and medium. Cells were incubated with 20 μCi/ml [^{3}H] glycine (specific activity 11.1 mCi/μmole) for 30 min and then 50 μg/ml unlabelled glycine was added. After 30 min and 150 min additional incubation, 1 ml samples were taken and the resuspended cell and medium fractions incubated at 37° for 2 hr in 1% SDS and 1% mercaptoethanol. The samples were than dialyzed overnight at room temperature against 0.1 M $NaPO_4$, pH 7.0, 0.1% SDS and 0.1% mercaptoethanol. One hundred and fifty μl aliquots were then electrophoresed on polyacrylamide gels. The gels were fractionated, solubilized and counted. Control (●-●-●); *cis*-hydroxyproline (o-o-o); dehydroproline (Δ--Δ--Δ); dehydrolysine (▲-▲-▲). Less than 70 counts appeared after fraction 20 in the cell gels and therefore they were not plotted for clarity of presentation. Calibration of molecular weight as a function of position in the gel was made by electrophoresing the following standard proteins and staining with Coomassie blue: lactic dehydrogenase (36,000), creatine kinase (40,000), pyruvate kinase (57,000), serum albumin (68,000), rat tail α-collagen (95,000).

Table 2
Pulse-chase with [^{3}H] glycine in the presence and absence of analogues.

Sample chase time (min)	Cells (cpm X 10^{-3})	Medium (cpm X 10^{-3})	Total (C + M) (cpm X 10^{-3})	$\frac{\text{cpm medium}}{\text{cpm total}} \times 100$ (%)
Control				
30	23.5	34.4	57.9	59.4
150	17.4	52.1	69.5	75.0
cis-Hypro				
30	34.0	4.2	38.2	11.1
150	30.4	6.9	37.3	18.4
Dehydropro				
30	18.5	2.8	21.3	13.1
150	16.9	5.6	22.5	24.9
Dehydrolysine				
30	24.8	6.3	31.1	20.2
150	26.6	12.2	38.8	31.5

Values in the cells and medium are from the experiment described in fig. 3.

containing significant amounts of any one of a number of proline analogues [5–8] or the lysine analogue, dehydrolysine [9], were secreted into the extracellular matrix at a markedly reduced rate. It was not possible to either adequately quantitate the amount of protein which was secreted or to qualitatively characterize it in any way. The present system of isolated fibroblasts which are rapidly synthesizing and secreting collagen has enabled such quantitation and qualitative characterization to be carried out. Dehm and Prockop [4] found with these cells that after 2 hr of incubation with [^{14}C] proline in the presence of 100 μg/ml of *cis*-hydroxyproline only 21% of the incorporated radioactivity was in the medium compared to 57% in the control medium. The present study showed that the abnormal collagens containing the proline analogues had a molecular weight identical to normal procollagen. Nevertheless, they accumulated intracellularly where they appeared to undergo slow degradation. These results are consistent with the notion that a critical amount of *trans*-hydroxyproline is required for normal secretion. All three analogues caused a large decline in the rate of [^{14}C] proline incorporation after 3 hr of incubation relative to the control cells. This relative decrease is more striking when it is considered that 50% of the available isotope was incorporated after 4 hr by the control cells. We would expect such a depletion to lead to a reduced incorporation rate in the control cells and have little effect on the cells in the presence of analogues where less than 20% of the isotope was incorporated. Experiments in which higher concentrations of proline are supplied to the cells are in progress but these must be performed with care since changing the proline concentration can lead to alterations in the relative incorporation of analogues and proline. These findings suggest that either i) the retention of the abnormal collagens decreased the rate of further collagen synthesis and possibly other proteins, or ii) protein synthesis is decreased by the synthesis of other defective proteins containing the analogues, or iii) some other unrelated toxic effect is operative.

A significant fraction of molecules synthesized in the presence of dehydrolysine were slowly secreted. Previous experiments with embryonic tibiae suggested that either hydroxylysine or glycosylated hydroxylysine were necessary for normal secretion but no estimate could be made of the minimal number of these residues which were required [9]. Because we have not been able to measure the degree of replacement of lysine by the analogues in the collagen in these cells we do not know whether this secretion was because of incomplete replacement or because the absence of hydroxylysine simply slows up secretion of the deficient molecules. The recent finding of a human genetic disease in which collagen lysine hydroxylase is either absent or markedly decreased in the dermis suggests that collagen secretion can take place even when the hydroxylysine content has been reduced to no more than one residue per triple chain tropocollagen molecule [15]. No estimate has been made of the rate at which secretion takes place in this defective tissue.

Acknowledgements

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