FURTHER CHARACTERIZATION OF EMBRYONIC TENDON FIBROBLASTS AND THE USE OF IMMUNOFERRITIN TECHNIQUES TO STUDY COLLAGEN BIOSYNTHESIS

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ABSTRACT

Morphological studies were carried out on fibroblasts from chick embryo tendons, cells which have been used in a number of recent studies on collagen biosynthesis. The cells were relatively rich in endoplasmic reticulum and contained a well-developed Golgi complex comprised of small vesicles, stacked membranes, and large vacuoles. Techniques were then devised for preparing cell fragments which were penetrated by ferritin-antibody conjuates but which retained the essential morphological features of the cells. Finally, the new procedures were employed to develop further information as to how collagen is synthesized. As reported elsewhere, preliminary studies with ferritin-labeled antibodies showed that prolyl hydroxylase was found in the endoplasmic reticulum of freshly isolated fibroblasts and that procollagen is found in both the cisternae of the endoplasmic reticulum and the large Golgi vacuoles. In the experiments described here, the cells were manipulated so that amino acids continued to be incorporated into polypeptide chains but assembly of the molecule was not completed because hydroxylation of prolyl and lysyl residues was prevented. The results indicated that these manipulations produced no change in the distribution of prolyl hydroxylase. Examination of the cells with ferritin conjugated to antibodies which reacted with protocollagen, the unhydroxylated form of procollagen, demonstrated that protocollagen was retained in the cisternae of the endoplasmic reticulum during inhibition of the prolyl and lysyl hydroxylases. Assays for prolyl hydroxylase with an immunologic technique demonstrated that although the enzyme is found within the endoplasmic reticulum, it is not secreted along with procollagen. The observations provided further evidence for a special role for prolyl hydroxylase in the control of collagen biosynthesis.

Recent studies have demonstrated that collagen is first synthesized as a precursor molecule consisting of three pro-α chains, which are larger than the α chains in collagen because of extensions at the NH$_2$-terminal ends (for review, see reference 1). The pro-α chains are assembled on membrane-bound ribosomes (2–4) and are modified by a series of posttranslational enzymic reactions which

include hydroxylation of selected prolyl residues, hydroxylation of selected lysyl residues, and glycosylation of some of the hydroxylysyl residues (for reviews, see references 1, 5). In addition, the three pro-α chains associate and are linked by interchain disulfide bonds (1, 5–10). The NH$_2$-terminal extensions on procollagen are then cleaved by a peptidase or several peptidases before the molecule can form normal extracellular collagen fibers (1, 11).

Fibroblasts isolated from chick embryo tendons and incubated in suspension have been used in a number of recent studies on the synthesis and secretion of procollagen (see references 1, 5, 6, 10, 12). One of the interesting observations which has been made with the tendon cells as well as several other types of cells is that the synthesis of procollagen can be manipulated so that amino acids continue to be incorporated into the polypeptide chains but assembly of the molecule is not completed. By incubating cells for 1 or 2 h under anaerobic conditions or with an iron chelator, the prolyl and lysyl hydroxylases which synthesize hydroxyproline and hydroxylysine in collagen are inhibited (13, 14). Under these conditions, the cells synthesize and retain protocollagen, the unhydroxylated form of procollagen (15). If inhibition of the hydroxylases is reversed after 1 or 2 h, the intracellularly retained protocollagen is hydroxylated and secreted (14–16). From these observations, it appears that determining the location of prolyl hydroxylase and protocollagen in the cells would provide important information as to which cellular organelles are involved in specific stages of the assembly and secretion of procollagen.

Earlier studies with peroxidase-labeled antibodies directed against prolyl hydroxylase and light microscopy demonstrated that the prolyl hydroxylase was distributed in localized regions of the cytoplasm (17). However, the resolution of the light microscope technique did not allow conclusions about the organelles containing the hydroxylase. Therefore we explored the use of labeled antibodies and electron microscopy to answer questions about the detailed localization of the enzyme.

Preliminary electron microscope studies with peroxidase-labeled antibodies suggested an association with the rough endoplasmic reticulum (unpublished observations). However, extensive deposits of the reaction product were seen throughout the endoplasmic reticulum and the resolution of this enzyme-labeled antibody technique was clearly not sufficient to decide whether the hydrox-

ylase was bound to ribosomes, membranes, or present in cisternae of the rough endoplasmic reticulum. Ferritin-labeled antibodies generally permit a more precise localization (18–20). We have therefore developed improved procedures for using such conjugates to study the biosynthesis of collagen in embryonic tendon cells. Preliminary reports on some of these studies have already been published (21, 22).

MATERIALS AND METHODS

Electron Microscopy of Tendon Cells

Cells were prepared from the leg tendons of 5 dozen 17-day old chick embryos as described previously (12). The procedure was modified slightly in that the enzymic conditions were adjusted to the wet weight of the tissue. For each gram of tissue, 2.5 mg of collagenase (Sigma Chemical Co., St. Louis, Mo.) and 0.2 ml of a 2.5% solution of trypsin (Grand Island Biological Co., Grand Island, N. Y.) in 2 ml of minimum essential medium were used. Digestion was continued for 40 min or until the tissue was largely dispersed but a few particles still remained. In order to make the cells taken for electron microscopy comparable to those used in previous biochemical studies, the cells were incubated for 3 h in modified Krebs medium containing 10% fetal calf serum before further processing.

For electron microscopy of control cells, the cells were fixed for 1 h at 4°C with 3% glutaraldehyde in 0.06 M sodium phosphate buffer, pH 7.3, containing 0.14 M sucrose. The cells were washed with 0.1 M sodium phosphate buffer, and postfixed for 1 h at 4°C with 1% osmium tetroxide in 0.06 M sodium phosphate buffer, pH 7.3, containing 0.16 M sucrose. After washing with 0.9% NaCl, the cell pellets were stained with 0.5% magnesium uranyl acetate (Polysciences, Inc., Warrington, Pa.) for 30 min at room temperature. The samples were dehydrated with ethanol and embedded in Araldite (TAAB Laboratories, Reading, England). Electron microscopy was carried out with a JEM-100B electron microscope.

Preparation of Ferritin-Conjugated Antibody to Prolyl Hydroxylase and Procollagen

Antisera to prolyl hydroxylase were prepared by injection of the purified enzyme into rabbits as described elsewhere (17). Initially, specific antibodies were isolated from the antisera with an immunoadsorption procedure using enzyme covalently bound to agarose (22). This procedure was subsequently found to be unnecessary, because the antisera were highly specific and the antibodies were present in relatively high titers. In later experiments, therefore, IgG was isolated from antisera by chromatography on o-(diethylaminoethyl)cellulose

(23) and used directly for conjugation with ferritin. Antisera to procollagen were prepared as described elsewhere (24). The ferritin-antibody conjugates were prepared with glutaraldehyde (procedure II in reference 25) and isolated by gel filtration (26).

Assays of Prolyl Hydroxylase in Tendon Fibroblasts

Cells were incubated for 3 h as described above and then ruptured by homogenization or N_2 cavitation (27). The homogenization was carried out by suspending 10^8 cells/ml in 0.1 ml M sodium phosphate buffer, pH 7.3, or in 0.1 M Tris-HCl buffer, pH 7.8 at 4°C, containing 0.2 M NaCl and 0.1 M glycine. For the immunologic assays either phosphate or Tris-HCl buffer was used; phosphate buffer was not used when enzymic activity was assayed because phosphate may chelate the iron necessary for the reaction. A Teflon and glass homogenizer and a constant torque motor were used for homogenization (22). The N_2 cavitation was carried out with the same concentration of cells in the same buffers, and the cell suspensions were equilibrated in 2.5-ml portions at a given pressure for 30 min in a pressure cell (Parr Instrument Co., Moline, Ill.) before decompression.

Enzyme protein was assayed by passive hemagglutination inhibition (22). The assays were repeated at least five times and mean values are reported here. The test was performed in disposable plastic trays (Linbro Chemical Co., New Haven, Conn., model IS MRC 96 with round-bottomed cups) with a procedure similar to that described by Eskeland et al. (28). 1 drop (30 μl) of antiserum directed against prolyl hydroxylase (diluted 1:1,000 to 1:4,000) was added to 2 drops (60 μl) of serial dilutions of cell homogenate or purified enzyme, and incubated for 45 min at 37°C. Then 1 drop of Formalin-treated sheep red blood cells (Grand Island Biological Co.) coated with prolyl hydroxylase (17) was added and the trays were incubated again for 45 min at 37°C. The end points on the plates were read between 1 and 3 h after the second incubation. The assay was standardized with purified prolyl hydroxylase, using a value for $A_{280}^{1\%}$ of 77.3.[1] The value for $A_{280}^{1\%}$ was based on concentration determined by amino acid analysis of the enzyme and was somewhat higher than the value which was used previously (29). The amounts of enzyme protein contained in tendon cells reported here are considerably higher than the amounts reported previously in initial stages of this work (22) because of improvements in the passive hemagglutination inhibition assay. The principal improvements consisted of the use of larger reaction volumes, the use of more concentrated antisera, and storage of the enzyme used for standardization in more concentrated solution (0.1 mg/ml) so that there were smaller losses during freezing, thawing, and transfer.

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[1] Berg, R. A., Y. Kishida, S. Sakakibara, and D. J. Prockop. Manuscript in preparation.

With these improvements, the reproducibility of the assay was greatly enhanced.

Enzyme activity was assayed by using $(Pro-Gly-Pro)_n$ as the substrate and measuring the amount of $[^{14}C]CO_2$ released form α-keto-$[1-^{14}C]$glutarate (29, 30). Enzyme activities were compared under standard conditions where the specific activity of pure enzyme was 4,200 U/mg[1] (29).

Radioimmune Assay of Protocollagen

$[^{14}C]$Protocollagen was prepared by incubating tendon fibroblasts with α,α'-dipyridyl and $[^{14}C]$proline, and the protein was extracted from the cells as described previously (15). The radioimmune assay was a double precipitation technique carried out by reacting the $[^{14}C]$proline-labeled protocollagen with purified rabbit IgG directed against procollagen (24), and then precipitating the complex with goat IgG directed against rabbit IgG (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.). A precipitation curve was developed by varying the amount of $[^{125}I]$iodine-labeled (31) rabbit IgG while keeping the antibody concentration fixed at 1 mg of goat anti-rabbit IgG in a total volume of 0.4 ml. The solution contained 0.05% bovine serum albumin and 0.135 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0, and the equivalence point was obtained with 10 μg of rabbit IgG. The nonimmune rabbit IgG was then replaced with 10 μg of specific antiprocollagen IgG produced in rabbits (24) or specific antiprocollagen IgG diluted with nonimmune rabbit IgG so that the total amount of rabbit IgG remained 10 μg IgG. The precipitation was carried out at 4°C for 6 h. The pellets were washed twice with buffer and then dissolved in 0.5 ml of 0.01 M HCl and counted in 20 ml scintillation fluid consisting of 30 g of 2,5-diphenyloxazole (PPO, Packard Instrument Co., Inc., Downers Grove, Ill.), and 100 mg of 1,4-bis-(2-[4-methyl-5-phenyloxazolyl]) benzene (dimethyl POPOP, Packard Instrument Co., Inc.), mixed in 5 liters toluene and 3 liters ethylene glycol monomethyl ether. The samples were counted in a scintillation counter at approximately 40% efficiency for ^{125}I. The procedure was checked in each experiment by adding $[^{125}I]$iodine-labeled rabbit IgG to insure that over 90% of the rabbit IgG had been precipitated. Maximal precipitation of $[^{14}C]$protocollagen was found by varying the amount of antiprocollagen in the assay from 5 to 10 μg.

Labeling of Cell Fragments with Ferritin-Antibody Conjugates

Cell fragments were prepared by fixing the cells with 1% formaldehyde in 0.06 M sodium phosphate buffer, pH 7.3, containing 0.14 M sucrose for 1 or 3 h at 4°C, and homogenizing the fixed cells with 30 or 60 strokes under the specific conditions described elsewhere (22). The fragments were isolated by centrifugation at 20,000 g for 10 min, and incubated with about 0.2 ml of ferritin-antibody conjugate (1 mg of ferritin and 0.2–0.3 mg of

IgG per ml), at 4°C for 24-65 h. After washing two or three times with 5 ml of 0.1 M sodium phosphate buffer, pH 7.3, the fragments were further fixed with 3% glutaraldehyde at 4°C for 1 h and postfixed with 1% osmium tetroxide before further processing for electron microscopy.

RESULTS

Electron Microscope Characterization of the Tendon Fibroblasts

The cells isolated from embryonic tendons were of relatively small size and about 5 μm in diameter. They were generally round in appearance with elongated processes extending from the surface of the cell (Fig. 1). Occasional blebbing of the cell surface was seen, probably as a result of the enzymic digestion used to isolate the cells. The cells were relatively rich in endoplasmic reticulum and contained a well-developed Golgi complex comprised of small vesicles, stacked membranes, and large vacuoles. Some of the large vacuoles contained a fine filamentous material; the filamentous material was also occasionally seen in dilated membrane stacks (Fig. 2). The cytoplasm contained a number of microtubules and microfilaments, and in some cells the microfilaments formed a thick bundle circumscribing the nucleus of the cell. Except for the more rounded shape, and the occasional blebbing, the cells were similar to cells seen in sections of intact tendons (not shown).

Characterization of Ferritin-Antibody Conjugates

Ferritin was linked to antibodies directed against prolyl hydroxylase and the ferritin IgG conjugates were isolated by gel filtration. The latter step made it possible to obtain conjugates which were free of polymers and free of unconjugated IgG (see accompanying paper [25]). The fractions containing antibody linked to monomeric and dimeric ferritin were pooled and tested by passive hemagglutination. The results (not shown) demonstrated that, within the sensitivity limits of this assay, the IgG-ferritin conjugates had the same antibody activity per milligram of IgG as the initial IgG used to prepare the conjugate (25).

Ferritin was also conjugated to antibody directed against procollagen. Before preparing the conjugates, a radioimmune assay was used to determine whether this antibody, prepared by immunizing rabbits with procollagen and purified by immunoadsorption (24), also reacted with pro-

tocollagen. The results indicated a specific reaction with the protocollagen (Table I) in that up to 65% of the [^{14}C]protein was precipitated in the assay. The failure to precipitate all the [^{14}C]protein is probably explained by the observation that the antibodies react specifically with the NH$_2$-terminal extensions of pro-α chains (24) if the disulfide bonds are intact and that protocollagen under the conditions employed here consists in part of partially degraded molecules. In the preparation of protocollagen used for the experiments described in Table I, only about 50% of the [^{14}C]protein consisted of pro-α chains which were linked by interchain disulfide bonds as tested by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (6, 7, 10). The [^{14}C]protein was not precipitated after predigestion of the samples with a specific bacterial collagenase, an observation which supported the conclusion that the precipitated [^{14}C]protein was protocollagen.

Preparation of Cell Fragments for Reaction with Ferritin-Antibody Conjugates

Initial experiments with the tendon fibroblasts were directed toward solving two problems: (a) fixation of the cells with a procedure which did not destroy the antigenicity of the protein under investigation, and (b) treatment of the cells so as to allow penetration of the conjugates into different compartments without loss of the essential morphological features of the cells. Preliminary data on the procedures for solving these two problems have been presented elsewhere (22, 26), but because the problems are critical for interpreting data obtained with ferritin-labeled antibodies in cells, they were explored in greater detail here.

As a first step, the amount of immunoreactive prolyl hydroxylase in the cells was measured using a passive hemagglutination inhibition assay. In reexamining methods of disrupting the cells, it was found that homogenization with 60 strokes under the conditions employed previously (22) did not make all of the protein available for reaction with antibody (Table II). Higher values of enzyme protein were obtained either after disruption of the cells with N$_2$ cavitation, or after homogenization in the presence of 0.1% Triton X-100 (Table II). Most of the enzyme released by homogenization in 0.1% Triton X-100 was in an active form. In contrast, most of the enzyme released by N$_2$ cavitation was inactive. The difficulty in releasing

FIGURE 1 Electron micrograph of a tendon fibroblast incubated under control conditions for 3 h. Note the irregular surface processes, and the Golgi complex close to the eccentrically placed nucleus. The section was contrasted with uranyl acetate and lead citrate. × 25,000.
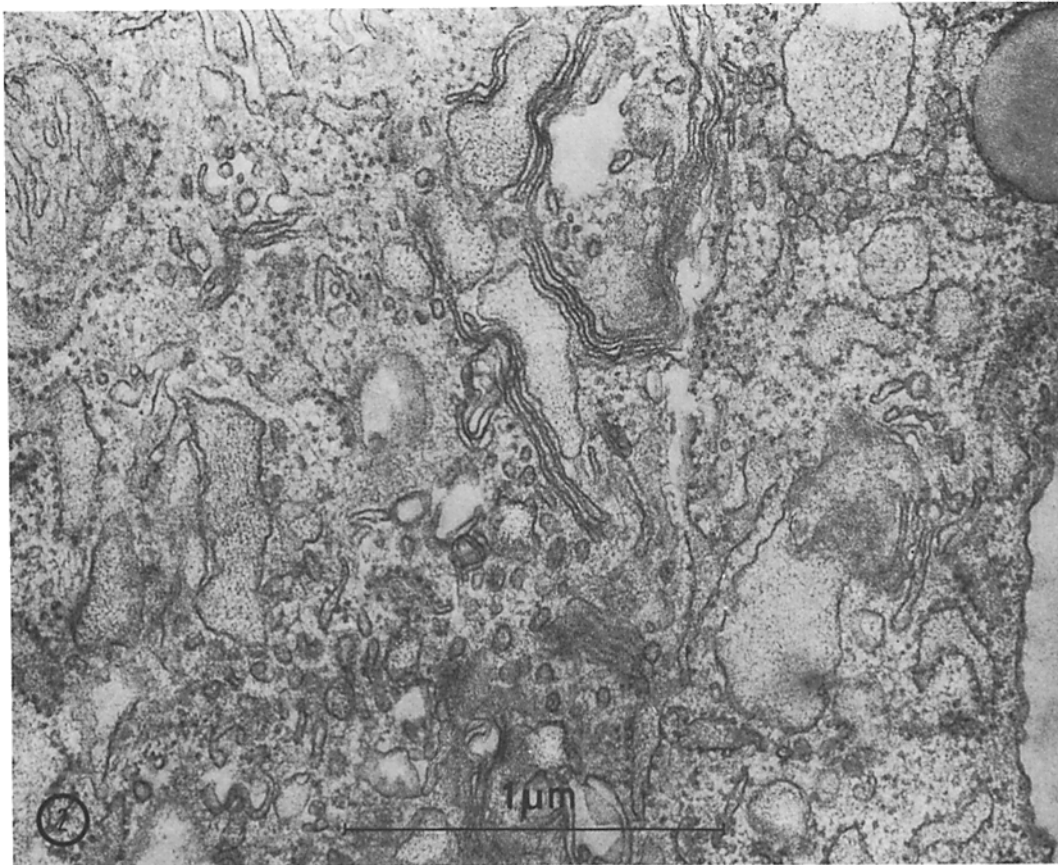
FIGURE 2 Electron micrograph showing part of the Golgi complex of a fibroblast incubated under control conditions for 3 h. Note the large smooth-surfaced vacuoles filled with filamentous material. The section was contrasted with uranyl acetate and lead citrate. × 50,000.

all the enzyme was inconsistent with initial conclusions that the enzyme was a soluble protein (32) but was consistent with more recent reports indicating that the enzyme is associated with membranous compartments of the cells (4, 22, 33).

As a second step, the effect of fixation on the antigenicity of prolyl hydroxylase was tested. In previous experiments with purified enzyme, fixation was found to reduce the amount of immunoreactive protein and this effect was greater with 1% glutaraldehyde than with 1% formaldehyde (22). Similar results were obtained here in experiments in which the effects of fixing cells with glutaraldehyde and formaldehyde were compared (Table III). Because of the deleterious effect of glutaraldehyde fixation on the amount of immunoreactive protein, it was not used in any further experiments. In contrast, fixation of the

cells with 1% formaldehyde for 1 h had no effect on the amount of immunoreactive protein observed after the cells were ruptured by N_2 cavitation (exp. 2 in Table II).

In the final stages of developing these procedures, a compromise was made between conditions which allow the antibody to penetrate into cellular compartments (see above) and conditions which preserved the morphological features of the cells. Treatment of the cells with detergents or N_2 cavitation destroyed all recognizable structures and therefore neither of these treatments could be used. Since fixation with 1% formaldehyde for 1 h did not decrease the amount of immunoreactive protein (Table II), cells were fixed with formaldehyde and then homogenized. Although fixation with formaldehyde was relatively brief, the enzyme did not diffuse out of the fragments in that only

TABLE I

Immunoprecipitation of [¹⁴C]Protocollagen with Antibodies to Procollagen

Experiment no.	IgG added	Pretreatment of protocollagen	Precipitate	
			[¹⁴C]Protein	Fraction of initial
			cpm	*%*
1*	Nonspecific	None	1,224	13
	Antiprocollagen	None	5,911	65
2‡, §	Nonspecific	None	400	4
	Antiprocollagen	Incubation with collagenase	425	4
	Antiprocollagen	Incubation without collagenase	2,972	31

* In exp. 1, 9,148 cpm of [¹⁴C]protocollagen was used for immunoprecipitation.
‡ In exp. 2, 9,455 cpm of [¹⁴C]protocollagen was used for immunoprecipitation.
§ In exp. 2 involving collagenase digestion of protocollagen, the collagenase was prepared by the method of Peterkofsky and Diegelmann (46). The incubation with collagenase was carried out in a volume of 0.5 ml containing 10 mM $CaCl_2$, 2.5 mM N-ethyl maleimide, and 50 mM Tris-HCl buffer, pH 7.4, for 90 min at 37°C.

TABLE II

Estimates of Total Amounts of Prolyl Hydroxylase in Chick Tendon Cells

Experiment no.	Procedure	Enzyme activity (μg equivalents/ 10⁸ cells)	Enzyme protein* (μg/10⁸ cells)
1	Homogenized, 60 strokes	12	24
	Homogenized, 110 strokes	12	45
	Homogenized, 60 strokes, in Triton X-100‡	40	60
	N_2 cavitation, 800 lb/in²	4	60
	N_2 cavitation, 1,600 lb/in²	2	60
2	N_2 cavitation, 800 lb/in²		60
	N_2 cavitation, 800 lb/in² after formaldehyde fixation §		60

* Twofold serial dilutions were used to assay enzyme protein by passive hemagglutination inhibition; the values presented here are means of five separate determinations.
‡ Homogenization was carried out in 0.1% Triton X-100 for the last 10 strokes.
§ Cells were first fixed for 1 h in 1% formaldehyde (see Table III).

about 10% of the immunoreactive protein was found in the supernatant fractions (Table III). However, antibody could diffuse into the cell fragments, since the passive hemagglutination inhibition assay indicated that about 44% of the available immunoreactive prolyl hydroxylase still reacted with the antibody (Table III). The ease with which macromolecules diffused into the fragments was further demonstrated in experiments with ferritin conjugated to nonimmune IgG. When the cell fragments were incubated with ferritin linked to nonimmune IgG and fixed without washing the fragments, ferritin was seen in compartments such as cytoplasm, endoplasmic reticulum, Golgi vacuoles, and nuclei (Fig. 3 *a* and *b*). The penetration of the ferritin conjugates into cell fragments apparently was correlated with membrane breaks. Since the homogenization produced a variable breakage of organelles, there was a considerable variability in the amount of ferritin seen in different compartments. In particular, penetrable Golgi vesicles and vacuoles were observed less frequently than penetrable cytoplasm and cisternae of endoplasmic reticulum. This problem, as discussed elsewhere,[2] can probably be explained by the difficulty of breaking the separate vesicles and vacuoles of the Golgi complex. When

[2] Nist, C., K. von der Mark, E. D. Hay, B. R. Olsen, P. Bornstein, R. Ross, and P. Dehm. Location of procollagen in chick corneal and tendon fibroblasts by ferritin-conjugated antibodies. Manuscript in preparation.

TABLE III

Availability of Enzyme Protein for Reaction with Antiprolyl Hydroxylase after Fixation and Homogenization of Chick Tendon Cells

		Enzyme protein (% of maximal)‡		
Fixation agent	Method of cell disruption	Supernate I	Supernate II	Cell fragments
Glutaraldehyde	Homogenization and N_2 cavitation, 800 lb/in²	1	< 1	2
Formaldehyde	Homogenization and N_2 cavitation, 800 lb/in²	2	10	88
Formaldehyde§	Homogenization	< 2	10	44

* Tendon cells were prepared as described in Materials and Methods. The cells were separated from the medium, fixed for 1 h with either 1% glutaraldehyde or 1% formaldehyde, centrifuged to separate supernate I from the fixed cells, homogenized, and then centrifuged to separate supernate II from the cell fragments. The cell fragments were cavitated at 800 lb/in² before being tested for enzyme antigen by passive hemagglutination inhibition.

‡ The percent of maximal value for enzyme portein was determined as in Materials and Methods and in Table II.

§ Tendon cells were prepared as above but not cavitated before being tested for enzyme antigen.

the fragments were washed with buffer before fixation, there was no ferritin in the fragments previously incubated with the ferritin linked to nonimmune IgG (Fig. 4).

Similar studies, reported elsewhere (26), were carried out using [¹⁴C]proline-labeled procollagen as the intracellular antigen. The results indicated that about 80% of the [¹⁴C]procollagen was recovered in cell fragments obtained by homogenizing cells which were fixed with 1% formaldehyde for 1 or 3 h.

Location of Prolyl Hydroxylase in Fibroblasts Treated with α,α'-Dipyridyl

As reported previously (22), experiments with ferritin-antibody conjugates demonstrated that prolyl hydroxylase was found in the cisternae of the endoplasmic reticulum of the fibroblasts examined under control conditions (Fig. 5). There was no indication that the immunoreactive enzyme protein was confined to the inner surface of the membrane of the endoplasmic reticulum, and no indication that there was a higher concentration near the membrane, but the results did not exclude the possibility that the enzyme may have been displaced from the inner surface of the membrane during the preparative procedures.

Because of the difficulty in breaking Golgi vesicles and vacuoles, it is difficult to interpret the lack of staining of the Golgi complex with antibodies to prolyl hydroxylase. However, even if our data to not provide conclusive evidence that prolyl hydroxylase is not present in the Golgi apparatus, it should be noted that even after examining a large

number of cell fragments obtained from different cell experiments, we have not been able to observe significant labeling of Golgi elements. On the other hand, by incubating cell fragments from similar cell experiments with antibodies to procollagen, it was always possible to observe labeling of Golgi vacuoles.

Radiochemical studies have shown that when tendon fibroblasts are incubated with appropriate concentrations of α,α'-dipyridyl, the cells rapidly accumulate protocollagen (12, 14–16). Since prolyl hydroxylase has been shown to bind tightly to protocollagen in stable enzyme-substrate complexes (34), we examined the question of whether accumulation of protocollagen leads to a change in the distribution of prolyl hydroxylase among or within cell compartments. Tendon fibroblasts were incubated for 3 h with 0.3 mM α,α'-dipyridyl, cell fragments were prepared as described above, and the fragments were incubated with ferritin linked to antiprolyl hydroxylase. The specific labeling with ferritin was the same as in control cells. Also, there was no distinctive pattern to the specific labeling seen in the cisternae of the endoplasmic reticulum (Fig. 6).

Location of Protocollagen in Cells Treated with α,α'-Dipyridyl

As indicated above (Table I), the antibodies directed against the amino-terminal extensions in procollagen also reacted with protocollagen. Therefore, the ferritin conjugates used previously for locating procollagen could be used here to study the distribution of protocollagen in cells incubated with α,α'-dipyridyl. As an initial part of
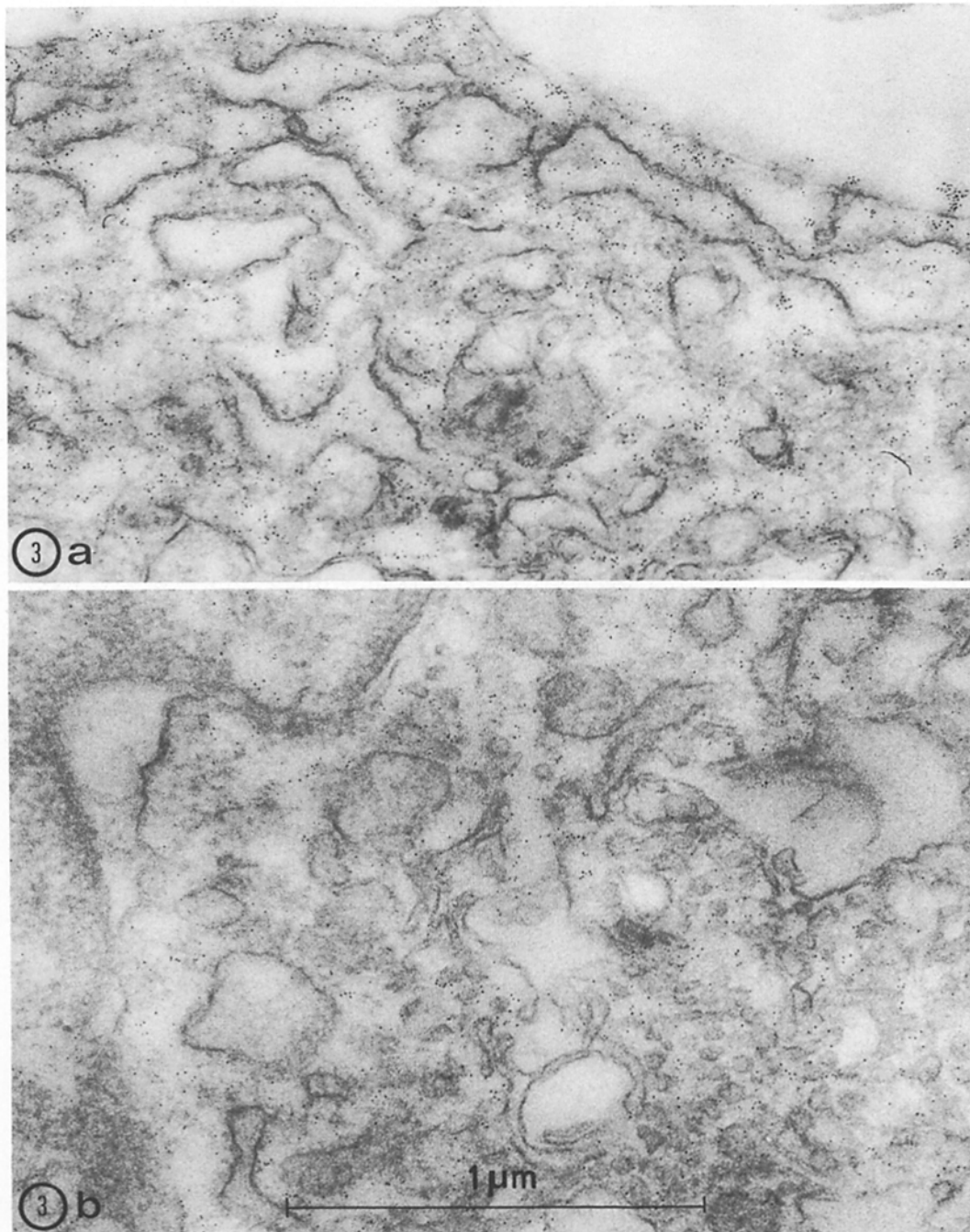
FIGURE 3 a and b   Electron micrographs showing penetration of ferritin-conjugated nonimmune rabbit IgG into different cell compartments in cell fragments. The cells were incubated under control conditions, fixed with 1% formaldehyde for 3 h at 4°C, and fragmented by homogenization as described in Materials and Methods. The cell fragments were then incubated with nonimmune rabbit IgG labeled with ferritin (1 mg of ferritin and 0.3 mg of IgG per ml) and fixed with 3% glutaraldehyde without washing the fragments after incubation with the ferritin conjugate. Note the presence of ferritin particles within the cisternae of the endoplasmic reticulum and cytoplasm (Fig. 3 a), as well as within the nucleus and the Golgi region of the cell (Fig. 3 b). The section was not stained. × 60,000.
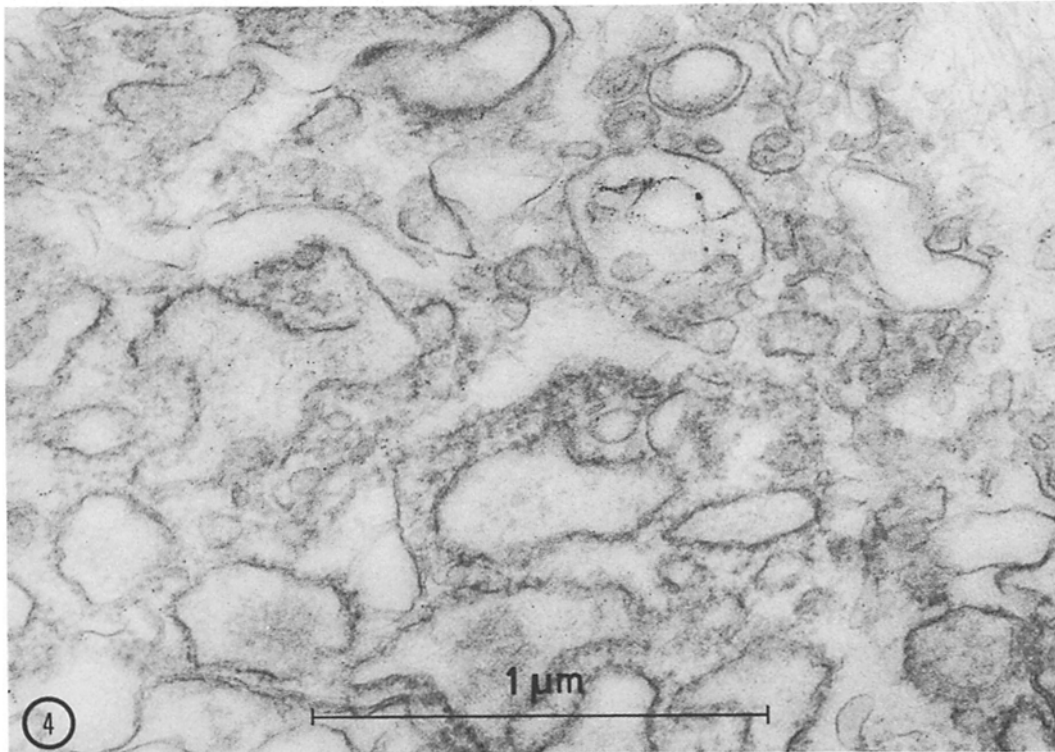
FIGURE 4 Electron micrograph showing part of a tendon fibroblast which was incubated under control conditions, fixed with 1% formaldehyde for 1 h at 4°C, homogenized with 30 strokes, and incubated with nonimmune rabbit IgG labeled with ferritin (1 mg of ferritin and 0.3 mg of IgG per ml). After incubation with ferritin conjugate the cell fragments were washed with buffer before fixation with 3% glutaraldehyde and further processing for electron microscopy. Note the absence of significant numbers of ferritin particles in different compartments, as compared with Fig. 3. The section was not stained. × 60,000.

these experiments, sections of whole cells were examined. After incubation with α,α'-dipyridyl, there was no significant change in the morphology of the cells. When fragments from the cells incubated with α,α'-dipyridyl were treated with ferritin conjugated to antiprocollagen, specific labeling was seen only in the cisternae of the endoplasmic reticulum (Fig. 7). There was no labeling of the cytoplasm or of membrane-bound ribosomes.

Failure of Prolyl Hydroxylase to be Secreted by Cells Synthesizing and Secreting Procollagen

Since prolyl hydroxylase was found in the cisternae of the endoplasmic reticulum and did not appear to be confined to the inner surface of the cisternal membrane, we examined the question of whether the enzyme is secreted together with procollagen. Tendon cells were incubated with

[^{14}C]proline and with or without α,α'-dipyridyl for 0–210 min. Hemagglutination inhibition assays indicated that the medium from the cell incubations contained a small amount of protein which reacted with antibodies to prolyl hydroxylase. Assay of the medium with immunodiffusion using an Ouchterlony technique (17) gave a reaction of complete identity with purified enzyme (not shown), indicating that the immunologically active material in the medium was in fact enzyme protein. However, the amount of enzyme protein in the medium from 10^8 cells was only about one-eighth the amount in the cells and the amount in the medium did not increase when the cells were incubated for 210 min under conditions in which they synthesized and secreted [^{14}C]procollagen (Table IV). Also, the amount of enzyme protein in the medium was the same when the cells were incubated with α,α'-dipyridyl so that the prolyl and lysyl hydroxylases were inhibited,
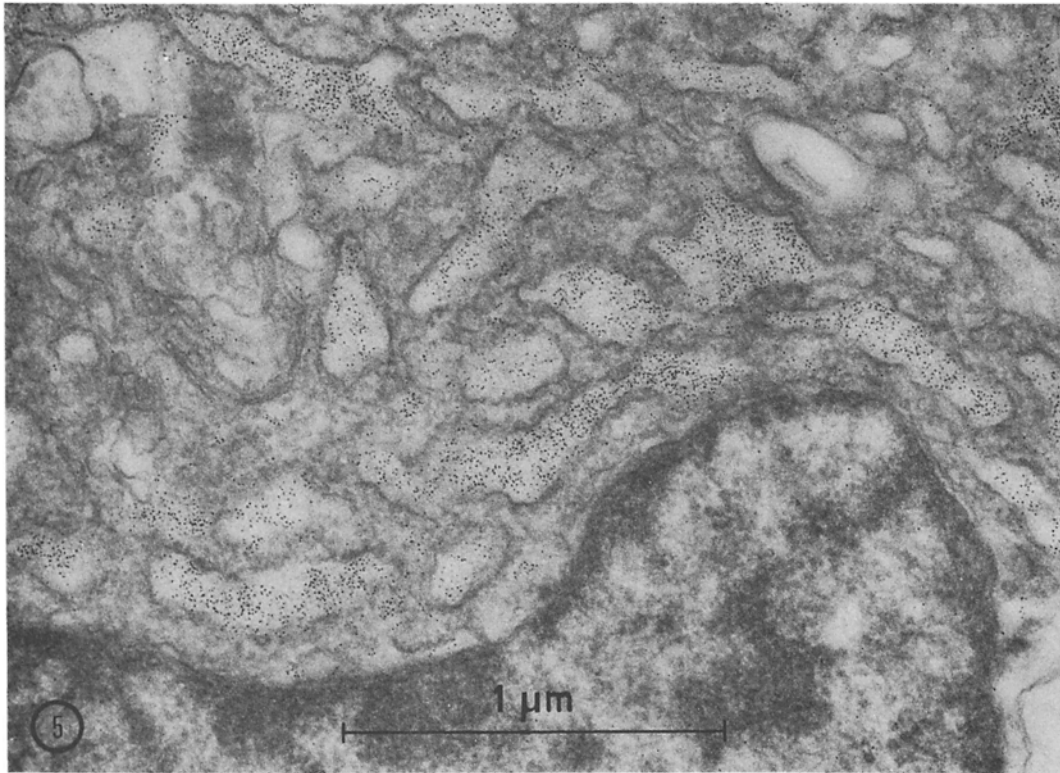
FIGURE 5 Electron micrograph showing part of a fragmented tendon fibroblast incubated with ferritin-labeled antiprolyl hydroxylase antibody. The cells were incubated under control conditions, fixed with 1% formaldehyde for 3 h at 4°C, homogenized, and incubated with the ferritin conjugate (1 mg of ferritin and 0.2 mg of IgG per ml) for 36 h at 4°C. Note the heavy labeling of the cisternae of the endoplasmic reticulum, and the absence of significant labeling of the cytoplasm except in places showing localized damage to the membranes of the endoplasmic reticulum. The section was not stained. × 50,000.

and the synthesis and secretion of procollagen was inhibited. The results indicated therefore that the enzyme was not secreted as the cells synthesized and secreted procollagen.

DISCUSSION

Several characteristics of the tendon fibroblasts used here simplify the use of ferritin-antibody conjugates to locate intracellular antigens in various organelles (10, 12, 15, 16, 26). The fibroblasts are free of extracellular matrix, and therefore homogenization of the cells cannot cause contamination of intracellular structures with extracellular materials. A second characteristic of the cells is that they represent a relatively homogeneous population of fibroblasts (12) and therefore there are no serious sampling problems in examining the fragments. A third favorable feature of the cell system is that it has been studied extensively with

biochemical techniques and the synthetic activity of the cells is well characterized (1, 10, 12, 15). In particular, it is known that the principal metabolic activity of the cells is the synthesis and secretion of procollagen, and that they therefore contain relatively large amounts of this protein and enzymes required for its synthesis (10, 12, 15, 16, 26).

Previously published techniques for using ferritin-antibody conjugates to locate specific intracellular components have included application of the conjugates to sections from cells embedded in cross-linked bovine serum albumin (18, 19), use of ultrathin frozen sections of nonembedded tissue (35) or subcellular fractions of cells (20, 36). In experiments preliminary to those reported here (unpublished observations), we encountered considerable nonspecific deposition of the conjugates on sections of tendon fibroblasts embedded in cross-linked bovine serum albumin. Also, it was

apparent that since only antigens in the surface of the sections were available for reaction with antibodies, the procedure was less sensitive than procedures which allow diffusion into cells or cell fragments. We found that reacting the conjugates with subcellular fractions of unfixed cells was unsatisfactory because it was difficult to prepare homogeneous subcellular fractions from the tendon fibroblasts and because it was difficult to determine whether antigens were displaced from organelles during their isolation.

The procedures described here avoided most of these difficulties and they probably can be applied to studying the distribution of a large number of antigens in the same cells. In addition, the procedures might well be applied to other cell types or to intact tissues if the procedures are suitably modified. In modifying the present procedures for other cells and tissues, however, it would be important to establish that the fixation procedure employed did not abolish the antigenic properties of the particu-

lar antigen under investigation. Also, it would be important to establish that the procedures allowed adequate penetration of the ferritin-antibody conjugates without destroying the essential morphological features of the cell. In the special case where the antigen under investigation is an enzyme, and where the ferritin-antibody conjugate is found in more than one compartment, it would also be important to determine whether the cells contain both active and inactive forms of the enzyme. This was not an important consideration in locating prolyl hydroxylase in the fibroblasts used here, since all the specifically bound ferritin was in one compartment. Also, assay of total antigen and enzyme activity suggested that most of the enzyme was in an active form. This latter situation is probably not obtained in all cells synthesizing collagen, since it has been demonstrated that some cultured fibroblasts contain inactive forms of prolyl hydroxylase which react with antibodies to the enzyme (37). It might be
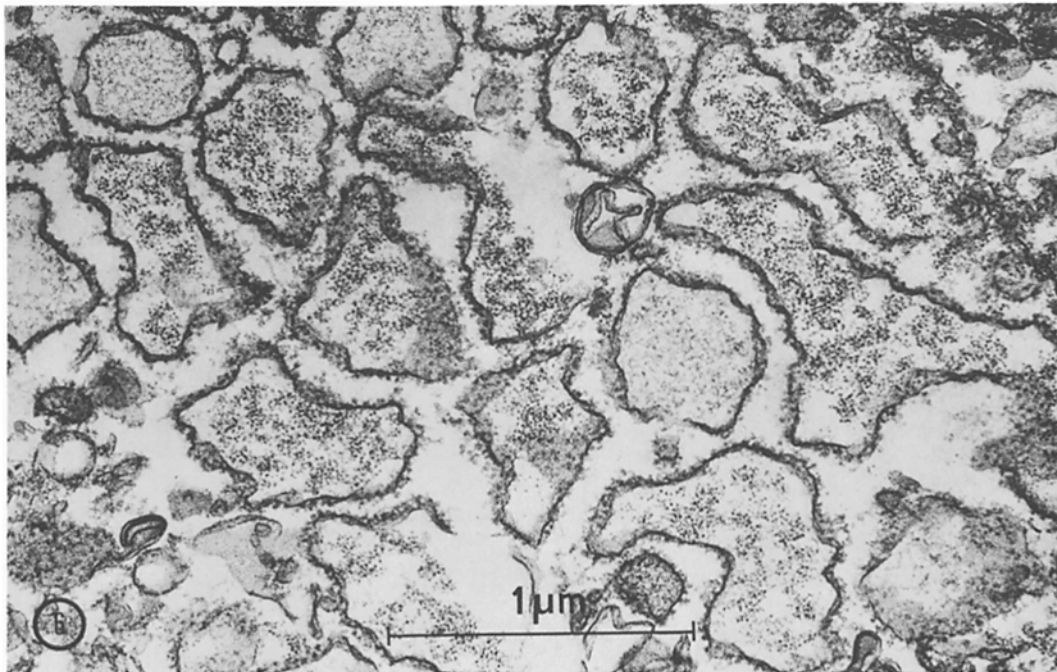

FIGURE 6   Electron micrograph of fragmented tendon fibroblasts incubated with ferritin-labeled antiprolyl hydroxylase. The cells were incubated at 37°C for 3 h in the presence of 0.3 mM α,α'-dipyridyl, fixed with 1% formaldehyde for 1 h at 4°C, and fragmented by homogenization. After incubation with ferritin-labeled antiprolyl hydroxylase (1 mg of ferritin and 0.2 mg of IgG per ml) for 24 h at 4°C, the fragments were washed and processed for electron microscopy. Note the labeling of the cisternae of the endoplasmic reticulum. Also note that fragmented microsomes show heavy labeling of the nonribosomal side of the endoplasmic reticulum membrane. The section was stained with alkaline bismuth (47). × 40,000.
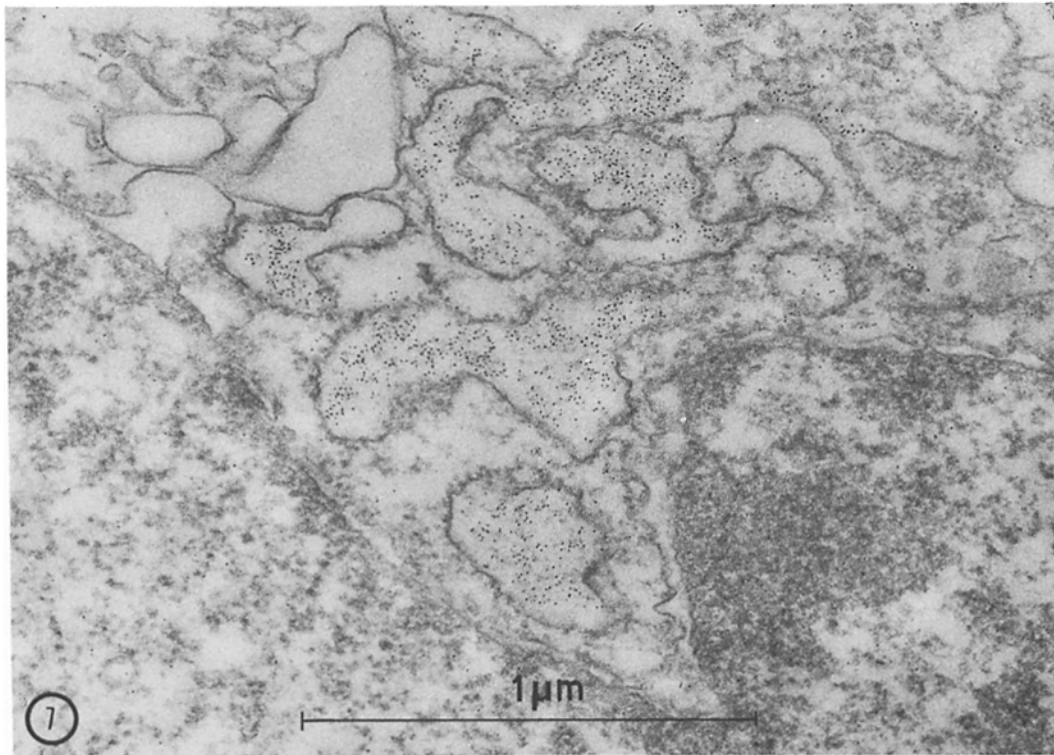
FIGURE 7 Electron micrograph of part of a fragmented tendon fibroblast labeled with antiprocollagen antibodies conjugated with ferritin. The cells were incubated at 37°C for 3 h in the presence of 0.3 mM α,α'-dipyridyl, fixed with 1% formaldehyde for 3 h at 4°C, and fragmented by homogenization. The fragments were incubated with ferritin-labeled antiprocollagen antibody (1 mg of ferritin and 0.3 mg of IgG per ml) for 65 h at 4°C and processed for electron microscopy. Note the labeling of the cisternae of the endoplasmic reticulum and the absence of ferritin labeling in the Golgi region of the cell (upper left-hand part of picture), and in the nucleus (lower part). The section was not stained. × 60,000.

noted that not all of the cisternae of the endoplasmic reticulum were labeled with the procedures described here and there are two possible explanations for this observation: One possibility is that various parts of the rough endoplasmic reticulum are highly specialized in their synthetic activity; another possibility is that the procedures do not make all parts of the endoplasmic reticulum equally accessible to the ferritin conjugates.

Previous studies with tendon fibroblasts incubated under control conditions demonstrated that prolyl hydroxylase is not in the cytoplasm of the cells and is not associated with ribosomes which are bound to the cytoplasmic side of the membrane of the endoplasmic reticulum (22). As reported here, after treatment of cells with concentrations of α,α'-dipyridyl which inhibit prolyl hydroxylase but do not inhibit polypeptide synthesis (12, 15,

16), prolyl hydroxylase remained in the cisternae of the endoplasmic reticulum and was not redistributed to another compartment as the cells accumulated protocollagen. Also, experiments with ferritin conjugated to antiprocollagen demonstrated that in the same cells the accumulated protocollagen was in the endoplasmic reticulum. The results, therefore, provided direct proof for the earlier suggestion that when the prolyl and lysyl hydroxylases are inhibited, protocollagen accumulates in the cisternae of the endoplasmic reticulum (22). From other studies with the cells used here[2] (26), it would appear that after the protocollagen is hydroxylated to procollagen, it moves to the Golgi vacuoles before secretion. This conclusion is consistent with recent observations in several different types of cells synthesizing collagen (38–40).

On the basis of previous observations indicating

that protocollagen is retained by cells, and that prolyl hydroxylase has a high affinity for protocollagen, it was suggested that prolyl hydroxylase may serve as a "barrier" or retention mechanism which prevents secretion of protocollagen (5). Stable enzyme-substrate complexes were readily isolated in vitro and such complexes dissociated when a sufficient number of prolyl residues in the protocollagen were hydroxylated (34). Also, it has been shown that protocollagen is nonhelical above 30°C and it cannot become helical at 37°C until it

is hydroxylated (41–43). The present observations indicating that protocollagen is retained in the cisternae of the endoplasmic reticulum, the same compartment which contains prolyl hydroxylase, are consistent with the assumption that the enzyme serves as at least one barrier or retention mechanism to prevent secretion of protocollagen until it is converted to procollagen. This scheme is further supported by the demonstration here that although prolyl hydroxylase is found within the endoplasmic reticulum, it is not secreted together with procollagen. The scheme makes it possible to explain the observations indicating that under some conditions, particularly long incubation periods, some protocollagen is secreted by cells (44, 45) since one would expect that increased intracellular concentrations of protocollagen should eventually saturate all the available enzymic sites. Further work will be necessary, however, to define more precisely the nature of the retention mechanism or mechanisms. In particular, it is not clear why the enzyme is not secreted together with procollagen (see reference 22).

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