LOCATION OF PROCOLLAGEN IN CHICK CORNEAL AND TENDON FIBROBLASTS WITH FERRITIN-CONJUGATED ANTIBODIES

CYNTHIA NIST, KLAUS VON DER MARK, ELIZABETH D. HAY, BJORN R. OLSEN, PAUL BORNSTEIN, RUSSELL ROSS, and PETER DEHM

From the Departments of Pathology and Biochemistry, University of Washington, Seattle, Washington 98195, and the Department of Biochemistry, The Rutgers Medical School, College of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854. Dr. von der Mark's present address is the Max-Planck-Institut für Biochemie, Martinsried, West Germany, Dr. Hay's present address is the Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115, and Dr. Dehm's present address is the Max-Planck-Institut für Zellbiologie, Wilhelmshaven, West Germany.

ABSTRACT

Three distinct antiprocollagen preparations were characterized and used in immunocytochemical staining of chick embryo corneal and tendon cells. The several ferritin-conjugated antibody preparations permitted similar location of procollagen in the cisternae of the rough endoplasmic reticulum and in Golgi elements in both cell types. The ability to demonstrate and interpret specific ferritin staining was dependent on the extent of membrane breakage in each of these organelles, coupled with adequate retention of cell morphology. Corneal fibroblasts appeared to suffer more extensive intracellular membrane damage under controlled conditions of homogenization than tendon fibroblasts, facilitating the identification of procollagen in Golgi vacuoles of these cells. None of the labeled material appeared to be cytoplasmic in origin since ferritin was observed in the cytoplasm only in the vicinity of Golgi elements that were extensively broken. This study extends previous immunological evidence for the presence of procollagen in the Golgi complex and calls attention to the problems to be encountered in locating the antigen in small Golgi vesicles and lamellae.

The intracellular transport of the secretory protein, procollagen, and the role of the Golgi apparatus in this function have been the subject of much discussion and study (3, 5, 11, 12, 24, 26–28, 34). Procollagen is unusual insofar as it is secreted by several distinct cell types. In fact, morphological differences in the extent and character of the Golgi apparatus among collagen-secreting cells may be part of the basis for the different interpretations regarding the mode(s) of intracellular transport and secretion of this protein (12, 34).

The question of whether passage of procollagen through the Golgi apparatus is obligatory in connective tissue cells was first approached by electron microscope autoradiography using [^{3}H]proline; the large Golgi vacuoles of chondrocytes were readily labeled (25), and location in the Golgi zone of fibroblasts was also observed (27). However, quantitative electron microscope autoradiographic studies gave conflicting results as to the amount of proline-rich protein passing from endoplasmic reticulum into the Golgi vacuoles of

several cell types (12, 27, 28, 34) and suggested that two intracellular pathways might exist for collagen transport (27, 28).

Recent advances in immunocytochemical staining have provided valuable information regarding the intracellular location of cell proteins (15, 17, 23). To be successful, these techniques require preservation of antigenicity of intracellular proteins, accessibility of these proteins to ferritin-conjugated antibody, and retention of cell ultrastructure during experimental manipulation. In one method (15, 20), the tissue was embedded in albumin and sections (approximately 600 Å) exposed to antibody, whereas in the other (23), prefixed, broken cells were exposed to antibody before embedding by routine methods. In both, affinity-purified, ferritin-conjugated antibody provided a specific marker for antigen location.

In a study of the intracellular location of procollagen in chick embryo tendon cells, using essentially the method of Kraehenbuhl and Jamieson (15) we found (unpublished results) that albumin embedding and ferritin-conjugated antibody staining of thin sections of these cells resulted in a high degree of nonspecific staining even with affinity-purified antibody. Better results have been obtained by routine embedding in epoxy resin after controlled homogenization of lightly fixed cells, and incubation of cell fragments containing morphologically recognizable but broken organelles with ferritin-conjugated antibody (23, 24). In the chick embryo tendon cell, Olsen and Prockop and coworkers showed that procollagen (24) and also proline hydroxylase (23) were located within the cisternae of the rough endoplasmic reticulum. Specific labeling of large Golgi vacuoles by antiprocollagen was observed, especially after colchicine treatment to increase their number (24), but labeled Golgi images in the cell fragments were relatively infrequent due, presumably, to limitation of entry of ferritin into Golgi elements (see Discussion).

In order to study the role of the Golgi apparatus in the intracellular transport of procollagen in more detail, we have applied ferritin-labeled antibodies to fragments of corneal fibroblasts. The corneal fibroblast of the 14-day chick embryo is particularly well suited for a study of this type, because the Golgi vacuoles are large (13) and the cells are engaged in no significant synthetic activity other than that involved in matrix production and normal turnover of intracellular proteins (cell division having ceased). Results obtained with chick tendon fibroblasts by use of antibodies to three different preparations or derivatives of procollagen are presented for comparison.

MATERIALS AND METHODS

Preparation of Antigens and Antibodies

Procollagen was extracted and purified from embryonic chick cranial bone by a modification of the method of Monson and Bornstein (21). The protein was further purified by chromatography on DEAE cellulose at 4°C (30) with an equilibration buffer of 0.1 M Tris-HCl, pH 7.5, containing 2 M urea, and a linear salt gradient from 0 to 0.3 M NaCl over a total volume of 600 ml. This procollagen (procollagen B),[1] mixed with complete Freund's adjuvant, was used to raise antibodies in rabbits (antiprocollagen B).

Antibodies to purified pro-α1 chain, obtained from acid-extracted chick cranial bone procollagen (32), were prepared as described previously (33). IgG was purified from antipro-α1 sera by DEAE cellulose chromatography (10), concentrated by ultrafiltration on a PM 30 membrane (Amicon Corp., Lexington, Mass.), and equilibrated with 0.2 M sodium borate, 0.15 M NaCl, pH 8.0. This γ-globulin preparation recognized both pro-α1 chains and intact cranial bone procollagen as determined by radioimmunoassays.

Affinity purification of antipro-α1 and antiprocollagen B antibodies was achieved by use of a procollagen immunoadsorbant. 7 mg of procollagen, purified by DEAE cellulose chromatography, were linked to 5 ml of CNBr-activated Sepharose 4B (6) with a coupling efficiency of 90%. DEAE-cellulose-purified IgG, containing antipro-α1 antibodies, was adsorbed to procollagen-Sepharose equilibrated with 0.2 M sodium borate, 0.15 M NaCl, pH 8.0, and specific antibodies were eluted with 3 M NaSCN in 0.1 M sodium phosphate, pH 6.0.

Procollagen T, secreted into the incubation medium of freshly isolated chick tendon fibroblasts, was partially purified by precipitation with $(NH_4)_2SO_4$ and extracted with acetic acid as described previously (7). Antisera to tendon fibroblast procollagen (antiprocollagen T) were then prepared in rabbits (7). Affinity purification of antiprocollagen T was achieved by using an immunoadsorbant containing a collagenase-resistant disulfide-bonded fragment (T3) which was derived from procollagen T (23). Specific antibodies (anti-T3) were eluted from the immunoadsorbant with 3 M NaSCN in 0.01 M sodium phosphate, pH 6.0.

[1] Procollagen extracted from cranial bone is referred to as procollagen B, and that from tendon fibroblasts as procollagen T.

Conjugation of Antibodies to Ferritin

Affinity-purified antibodies (anti-T3) isolated from antisera against procollagen T, and affinity-purified antibodies (antipro-α1) against pro-α1 chains of acid-extracted chick cranial bone procollagen were conjugated to ferritin with glutaraldehyde (23, 24). Ferritin-labeled antibodies, tagged with [^{125}I]iodine (18) to monitor recovery, were isolated by chromatography on 6% agarose (23, 24). The ferritin-antibody conjugates were then concentrated by ultrafiltration to about 1 mg ferritin and 0.3 mg of antibody per ml in 0.1 M Tris-HCl buffer, pH 7.5, and used for labeling of cell fragments.

Sheep antirabbit IgG $(Fab')_2$ was prepared by pepsin digestion (9) and purified by affinity chromatography on a rabbit IgG-Sepharose column. Purified $(Fab')_2$ labeled with [^{125}I]iodine was coupled to ferritin as described above and the ferritin conjugate purified by chromatography on a column of 4% agarose equilibrated with 0.1 M Tris-HCl, pH 7.5.

Radioimmunoassays

Direct binding immunoassays were performed by a modification of the assay described by von der Mark et al. (33). Antisera were absorbed with an excess of unlabeled chick α1 and α2 chains before addition of the labeled antigen. With higher volumes of rabbit antisera, 0.5 ml of sheep antirabbit IgG serum was used to insure complete precipitation of the antigen-antibody complex. The assay was run at room temperature, and heat denaturation of test antigens was carefully avoided.

Preparation of Corneal and Tendon Fibroblasts

Corneas from eight dozen 14-day chick embryos were suspended in 10 ml of Dulbecco-Vogt medium containing glutamine and equilibrated under 5% CO_2, 95% O_2 at 37°C. To the suspension were added 11 mg pure collagenase (Worthington CLSPA, Worthington Biochemical Corp., Freehold, N. J.), 20 mg crude collagenase (Worthington CLS), 1.1 ml 2.5% trypsin (Grand Island Biological Co., Grand Island, N. Y.), and 5 U chondroitinase ABC (Miles Laboratories, Inc., Kankakee, Ill.), and incubation was continued for 90 min, at which time the stromas had largely disintegrated. The cell suspension was passed through a nylon net and centrifuged at 600 *g* for 6 min. The resulting pellet was washed twice with 5 ml 10% fetal calf serum in phosphate-buffered saline, pH 7.4. Cells were resuspended in the same solution and counted.

Tendon fibroblasts from 17-day chick embryos were isolated by incubation of tendons from 60 embryos with trypsin and purified bacterial collagenase as described (8), except that Dulbecco-Vogt medium with glutamine was used instead of minimum essential medium. The cells were washed with 10% fetal calf serum as above.

Suspensions of either corneal or tendon fibroblasts were pelleted, resuspended in "NCI" medium (Grand Island Biological Co.) with 10% fetal calf serum, and incubated under O_2-CO_2 at 37°C for 3 h. Cells were pelleted and washed twice with 2 ml phosphate-buffered saline, pH 7.4, and then fixed with 1% formaldehyde in 0.06 M sodium phosphate buffer, pH 7.5, containing 0.14 M sucrose for 3 h on ice. Cells were centrifuged at 600 *g* for 3 min, washed twice with 2 ml of 0.1 M sodium phosphate, pH 7.5, and left overnight at 4°C in the same buffer. The cells were then fragmented by homogenization with a motor-driven glass-Teflon tissue homogenizer (Tri-R model S63C, Tri-R Instruments, Inc., Santa Monica, Calif.) at a setting of 5. The homogenate was chilled on ice every 10 strokes. Homogenization was continued in this manner until half of the cells appeared damaged in the light microscope (60 strokes for corneal fibroblasts, 100 strokes for tendon cells).

The suspension of cell fragments was centrifuged at 20,000 *g* for 10 min at 4°C, and incubated for 24 h at 4°C with 0.2 ml of ferritin-conjugated antibody. The corneal fibroblast fragments were labeled by a direct method employing ferritin-conjugated anti-T3. Tendon fibroblasts were treated either with a direct method or with an indirect method employing unlabeled antibody in the first step and ferritin-conjugated sheep $(Fab')_2$ directed against rabbit IgG as the second-step reagent. For the first step, either antiprocollagen B, anti-T3, or antipro-α1 antibodies were used.

Control fragments (tendon cells) were incubated with similar concentrations of ferritin-conjugated nonimmune rabbit IgG; one pellet was subsequently washed with buffer, one was not. The frequency of ferritin images in both controls was very low.

The fibroblast fragments, after incubation in immune or nonimmune ferritin-conjugated IgG, were washed twice with 5 ml 0.1 M sodium phosphate, pH 7.5, fixed on ice for 1 h with 3% glutaraldehyde in 0.06 M phosphate buffer containing 0.14 M sucrose, and osmicated with 1% osmium tetroxide in 0.06 M sodium phosphate buffer, pH 7.5, containing 0.16 M sucrose, on ice for 1 h. The fragments were rinsed thoroughly in normal saline, stained en bloc with 0.5% uranyl acetate in distilled water for 30 min, and embedded in Araldite. Sections were viewed unstained or stained with lead citrate or alkaline bismuth subnitrate.

RESULTS

Specificity of Antisera

When fragments from corneal cells were incubated with anti-T3 or tendon fibroblast fragments were incubated with either anti-T3, antiprocolla-

gen B, or antipro-α1, the labeling of the endoplasmic reticulum and the Golgi complex were essentially the same. Differences in specificities of the antibodies were observed in immunoassays (see below), but in view of the difficulty inherent in establishing quantitative differences by electron microscopy and the overlapping specificities of the antibody preparations, the similar reactivity of these preparations, as judged by electron microscopy, is not surprising.

The specificity of the antisera used in this study was compared in a series of direct binding immunoassays (Figs. 1–4). When cranial bone procollagen (procollagen B) was used as an antigen, several of the antisera (antiprocollagen B, antiprocollagen T, and antipro-α1) were effective in combining with the antigen (Fig. 1). Affinity-purified antibodies to a collagenase-produced derivative of procollagen T (anti-T3) were less effective as immunoprecipitants. This was not due to a lower concentration of IgG in the anti-T3 preparations, since anti-T3 antibodies at the same dilution effectively recognized tendon procollagen (Fig. 2). Thus, the specificity of anti-T3 preparations is restricted to determinants which are well represented on procollagen T but are not so prevalent on procollagen B. Conversely, antipro-α1 sera recognized procollagen B to a greater extent than procollagen T (Figs. 1, 2). These results were confirmed when pro-α1 and T3 were used as antigens (Figs. 3, 4). Antiprocollagen T antiserum, and the subset of antibodies present in the anti-T3 preparation, were not so effective as antiprocollagen B or antipro-α1 in combining with the pro-α1 chain (Fig. 3), whereas the T3 antigen contained no antigenic determinants capable of recognition by antipro-α1 serum (Fig. 4).

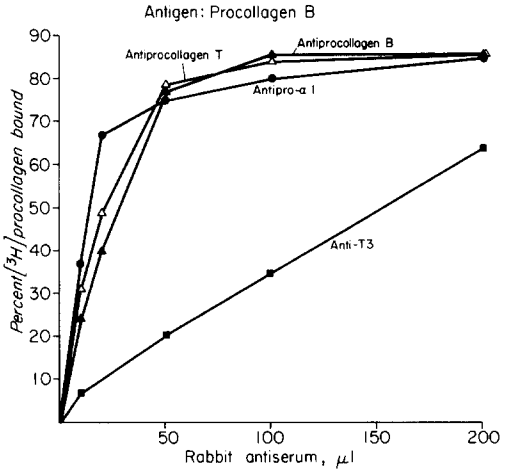


FIGURE 1. Titration curves using [^{3}H]procollagen B as test antigen.[1] Antisera used were: ▲——▲, antiprocollagen B (1:10 dilution); Δ——Δ, antiprocollagen T (1:5 dilution); ●——●, antipro-α1 (1:5 dilution); ■——■, anti-T3 (1:5 dilution). The assay was performed by adding the appropriate volume of diluted antiserum to 0.1 ml of a buffer containing 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5, 0.01% sodium azide, 1% ovalbumin, and 10 μg each of purified chick α1 and α2 chains. After incubation for 1 h at room temperature, test antigen (4,000–6,000 cpm dissolved in the above buffer) was added and incubation continued for an additional hour. Sheep antirabbit IgG serum (0.2–0.5 ml) was then added and precipitation allowed to occur overnight at 4°C. The precipitate was then centrifuged, washed once with 2 ml of 0.15 M NaCl, and counted in 10 ml of Aquasol. All assays were performed in duplicate and controls using nonimmune rabbit serum were performed to determine the extent of nonspecific precipitation of test antigens.

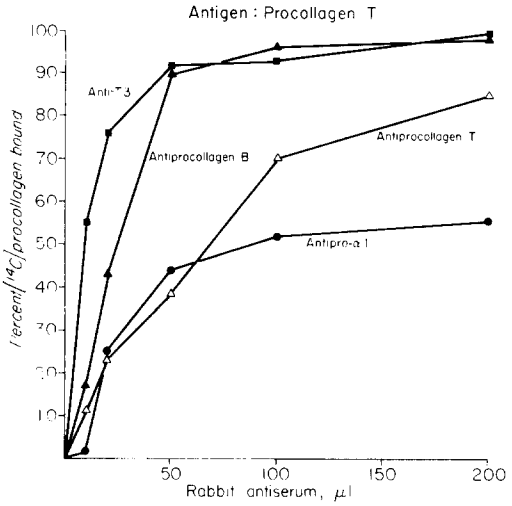


FIGURE 2. Titration curves using [^{14}C]procollagen T as test antigen.[1] Antisera used were: ▲—▲, antiprocollagen B (1:25 dilution); Δ——Δ, antiprocollagen T (1:50 dilution): ●——●, antipro-α1 (1:10 dilution); ■——■, anti-T3 (1:5 dilution). See legend to Fig. 1 for additional details.

Immunocytochemistry

Both corneal and tendon fibroblasts have a well-developed rough endoplasmic reticulum, with closely packed interconnected cisternae, often in parallel array. Corneal fibroblasts with plasma membranes broken by the partial homogenization usually show labeling of a few cisternae of endoplasmic reticulum by antibody to procollagen. In

some cases, all of the cisternae in a plane of section are labeled (endoplasmic reticulum, Fig. 5). For the antibody to penetrate the lumen of the cisterna and bind to procollagen, the membrane of the reticulum must be broken at some point (e.g., small arrow, Fig. 5), allowing diffusion of ferritin-conjugated antibody into the reticulum. Visible binding sites are uniformly distributed over regions of moderately dense material located within the cisternae (Figs. 5 and 6).

The distribution of antibody within the cisternae of the rough endoplasmic reticulum is similar in tendon and corneal fibroblasts (Figs. 5–8). Moreover, in tendon fibroblasts each of the three antibody preparations examined binds to intracisternal contents in similar fashion. Each of the ferritin-labeled antibody preparations labels the contents of the nuclear envelope (*pc*, Fig. 5; circular inset, Fig. 5), provided that the outer perinuclear membrane is broken.

We examined unstained sections in order to judge the extent of ferritin localization over the nucleus or cytoplasm (Fig. 5). Such background was negligible and when present was correlated with the presence of extensive breaks in the endoplasmic reticulum or Golgi elements. When breaks in Golgi elements were particularly extensive, the released stained procollagen over nearby organelles might lead to the appearance of an association that is not meaningful. When background cytoplasmic staining was entirely absent, no organelle membrane breaks were visible, even though the plasmalemma was broken. The specificity of the binding of antibody to procollagen was verified by controls employing nonspecific antibody (ferritin-conjugated rabbit IgG). Little or no ferritin was seen in these preparations, whether cell pellets were washed after exposure to antibody or not.

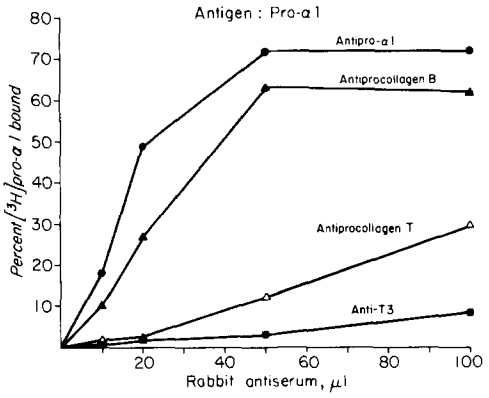


FIGURE 3. Titration curves using [^{3}H]pro-α1 as test antigen. Antisera used were: ▲——▲, antiprocollagen B (1:5 dilution); Δ——Δ, antiprocollagen T (1:5 dilution); ●——●, antipro-α1 (1:5 dilution); ■——■, anti-T3 (1:10 dilution). See legend to Fig. 1 for additional details.

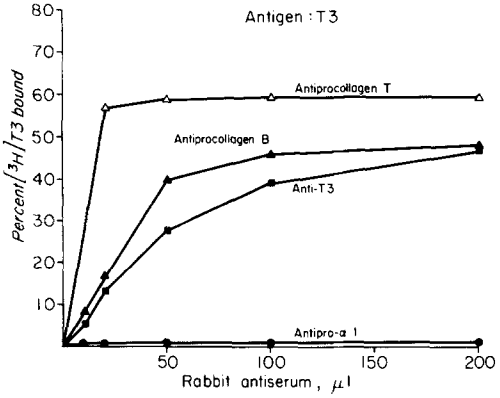
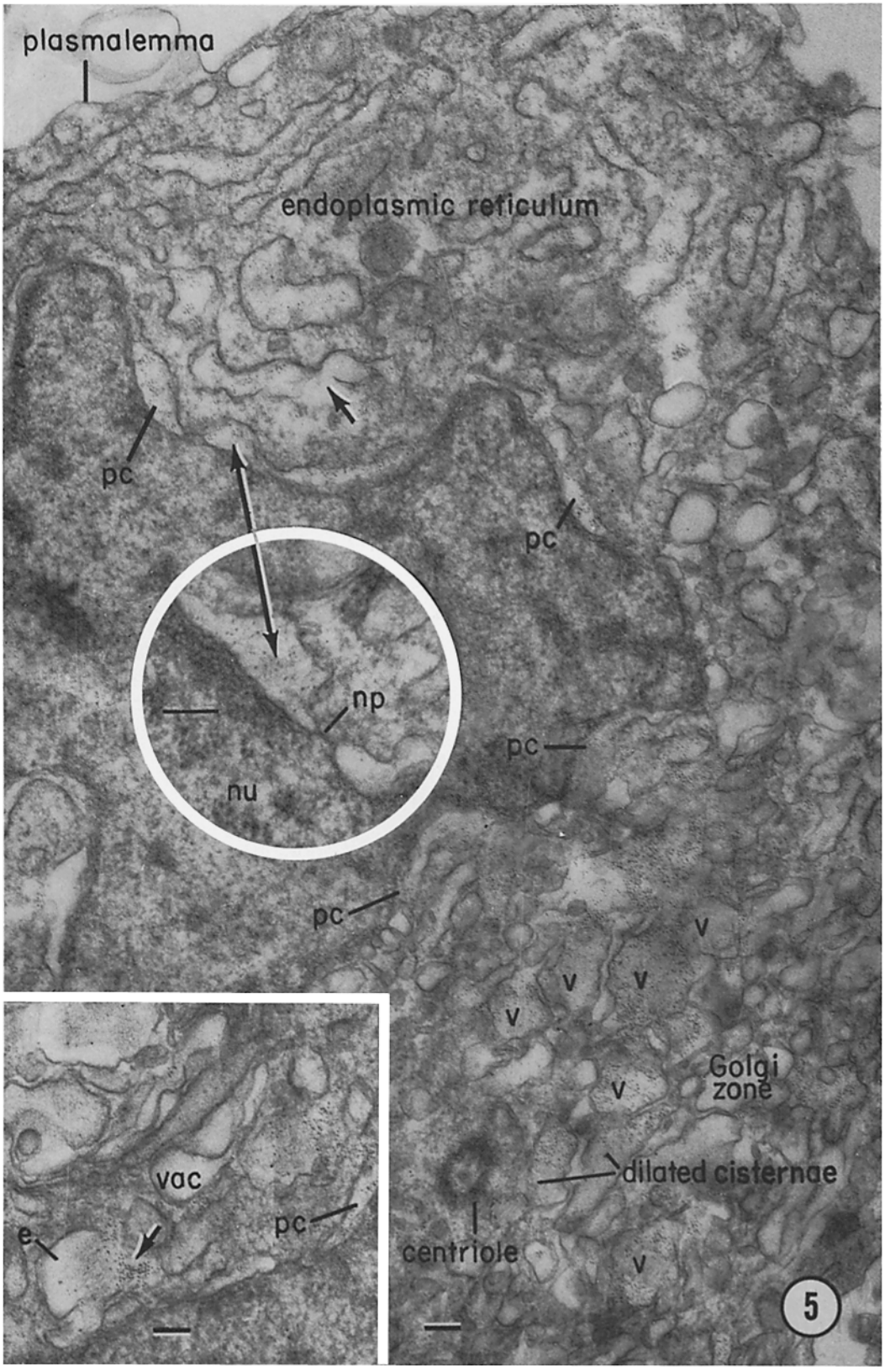


FIGURE 4. Titration curves using [^{3}H]T3 as test antigen. Antisera used were: ▲——▲, antiprocollagen B (1:25 dilution); Δ——Δ, antiprocollagen T (1:5 dilution); ●——●, antipro-α1 (1:5 dilution); ■——■, anti-T3 (1:10 dilution). See legend to Fig. 1 for additional details.

The Golgi complexes are well developed in both cell types, and contain numerous vesicles (from 25 to 175 nm in diameter) and flattened lamellae. Large vacuoles (200–300 nm in diameter) appear more common in the corneal fibroblast, a cell that produces more glycosaminoglycans than the tendon fibroblast (4, 13).

It was possible in sections of both corneal and tendon fibroblasts to find Golgi regions containing ferritin-conjugated antibody to procollagen regardless of which of the three antibody preparations was used. The homogenization technique, however, produced a distribution of organelle breakage so that penetrable Golgi vesicles and vacuoles were observed less frequently than penetrable cisternae of endoplasmic reticulum, and usually only in cells in which both organelles were extensively broken.

Labeled antibody was more commonly observed in the large vacuoles of corneal and tendon fibroblasts than in the smaller vacuoles and vesicles, presumably because it is easier to break the membranes of the larger elements without completely dispersing their contents (Figs. 8, 9). The contents of these large vacuoles tend to have moderate electron density (Figs. 8, 9). Whereas the cisternal space of the endoplasmic reticulum is


plasmalemma
endoplasmic reticulum
pc
pc
pc
pc
np
nu
v
v
v
v
v
Golgi zone
dilated cisternae
centriole
vac
e
pc
5

more or less uniformly labeled (*er*, Fig. 8), large vacuoles often have antibody staining only on the periphery of their dense contents (*v5*, Fig. 8). However, in the corneal fibroblast preparations, penetration of antibody into both the central and peripheral portions of large vacuoles was also observed.

The difficulties in disrupting membranes of the Golgi apparatus to permit entry of labeled antibody result in a variable amount of antibody within vesicles and vacuoles. To obtain good labeling in the smaller vacuoles and vesicles of the Golgi apparatus, it is necessary to severely damage the cell by homogenization. Thus, it is not surprising that electron-dense contents disappear completely from some profiles, that some Golgi zones disintegrate to form myelin figures, and that antibody-binding materials are found outside the vesicles and vacuoles. In Golgi zones that are not sufficiently fragmented to permit labeling of the contents of the vacuoles, there is no background staining, even though both the plasmalemma and the endoplasmic reticulum may be extensively broken in such cells.

It was difficult to detect antigen in Golgi lamellae; however, occasional membrane breaks (*x*, Fig. 10) may represent points of entrance of antibody to a lamella and adjoining dilated Golgi cisternae (see also Fig. 5, lower right). The amount of staining in disintegrated Golgi zones suggests that the intact organelle is relatively rich in procollagen.

DISCUSSION

Specificity of Antisera

It is now clear that the pro-α1 chain obtained from acid-extracted cranial bone procollagen represents a truncated precursor chain (21). Cleavage of disulfide-bonded regions occurs, presumably catalyzed by acid proteases released during homogenization of bone. Antibodies to pro-α1 are therefore directed to determinants on a restricted region of the precursor chain, determinants which may be lacking or not readily available in T3 (Fig. 4). Similarly, the procollagen T antigen used for preparation of antiprocollagen T and anti-T3 preparations was degraded to a greater extent than procollagen B (unpublished results). Thus a different spectrum of antibodies might be expected in response to the two antigens, and only a fraction of antiprocollagen B antibodies would be expected to combine with the T3 peptide fragment of procollagen.

Comparative studies of antibody specificity were performed in the hope that differences in specificity might be exploited in the intracellular location of procollagen. Thus, if structural modification in procollagen leading to intermediate forms occurred during transcellular movement, different antibody preparations, labeled with ferritin, might preferentially stain different subcellular compartments or organelles. Despite the significant quantitative differences in specificity of the several antibody preparations used (Figs. 1–4), such differences were in fact not observed in tendon fibroblasts. In view of the inherent difficulty in establishing quantitative differences by electron microscopy and the overlapping specificities of the antibody preparations, the similar reactivity of these preparations is perhaps not surprising. Other experiments (2, 16, 29) strongly suggest that the conversion of procollagen to collagen occurs extracellularly.

FIGURE 5. Electron micrograph of a section of a corneal fibroblast fragment from a formaldehyde-fixed, homogenized pellet treated with ferritin-labeled anti-T3 and washed to remove unbound antibody. The mechanical shear this cell received removed only part of the plasmalemma, so the cell remained intact even though most of the membranes of its Golgi vacuoles, Golgi cisternae, and endoplasmic reticulum were broken sufficiently (as at the arrow) to admit antibody. Ferritin molecules (electron-dense core, 45 Å) are visible within the endoplasmic reticulum and perinuclear cisterna (*pc*), in the cytoplasm where complete breakage of cisternae or vacuoles has occurred, and in most Golgi vacuoles (*v*) visible here. The plasmalemma is bare. A uranium precipitate, but no ferritin, can be detected in the nucleus. Unstained section (en bloc uranyl acetate). × 60,000. *Square inset:* the Golgi vacuoles that appear empty (*vac*) seem to have lost their moderately electron-dense content. One (*e*) has only partially lost its content (arrow) into the adjacent cytoplasm. × 60,000. *Circular inset:* the perinuclear cisterna in a tendon fibroblast labeled with antipro-α1 shows the same labeling pattern as that in the corneal fibroblast. *np*, nuclear pore; *nu*, nucleus. × 90,000. The bar is equivalent to 0.1 μm in all electron micrographs.

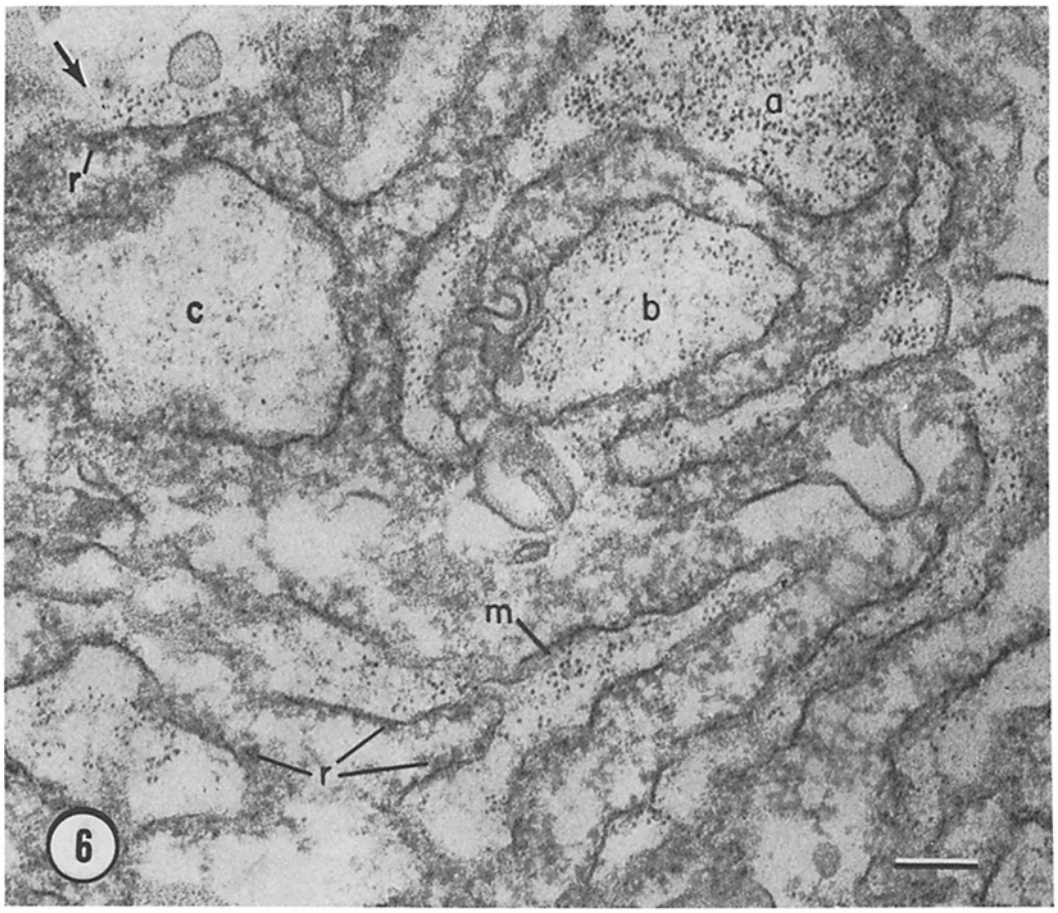


FIGURE 6. Higher magnification electron micrograph showing to better advantage the endoplasmic reticulum of a corneal fibroblast treated with anti-T3 antibody. Breaks in the membrane of the endoplasmic reticulum have permitted antibody to enter and move through the reticulum, labeling large numbers of intact cisternae that presumably are connected to one another. Cisternae *b* and *c* are lower in label density than cisterna *a*. At the upper left, there appears a single cisternal membrane which retains label (arrow) on its original inner side. Label is not present on the ribosomal side (*r*). In the lower middle, the ribosomal side (*r*) may again be compared with the intracisternal side (*m*) of the reticulum. Stained with alkaline bismuth subnitrate to enhance density of ferritin. × 115,000.

The Golgi Apparatus

The importance of the Golgi apparatus in the intracellular packaging, transport, and metabolism of secretory proteins is well recognized in several exocrine glands (14, 22). Its importance has been the subject of debate in the case of procollagen, since there is not always an obvious system of condensed secretory granules present, and since there have been indications from autoradiography that, in fibroblasts at least, transport through the Golgi apparatus would have to be rapid to account for the relatively small proportion of [^{3}H]proline-labeled product found in this organelle (27, 28).

Our results demonstrate that procollagen is found within all the secretory vacuoles of the Golgi complex of corneal fibroblasts during the time of greatest synthetic activity by these cells. The fact that labeling of the Golgi elements of tendon fibroblasts is less extensive (24) appears to be directly related to the ability of the antibody to penetrate the Golgi. The specificity of this technique offers an advantage over previous autoradiographic results; however, the limitations of the antibody technique make negative results (19) difficult to interpret.

When mechanical forces are applied to the cell during homogenization, each cell compartment

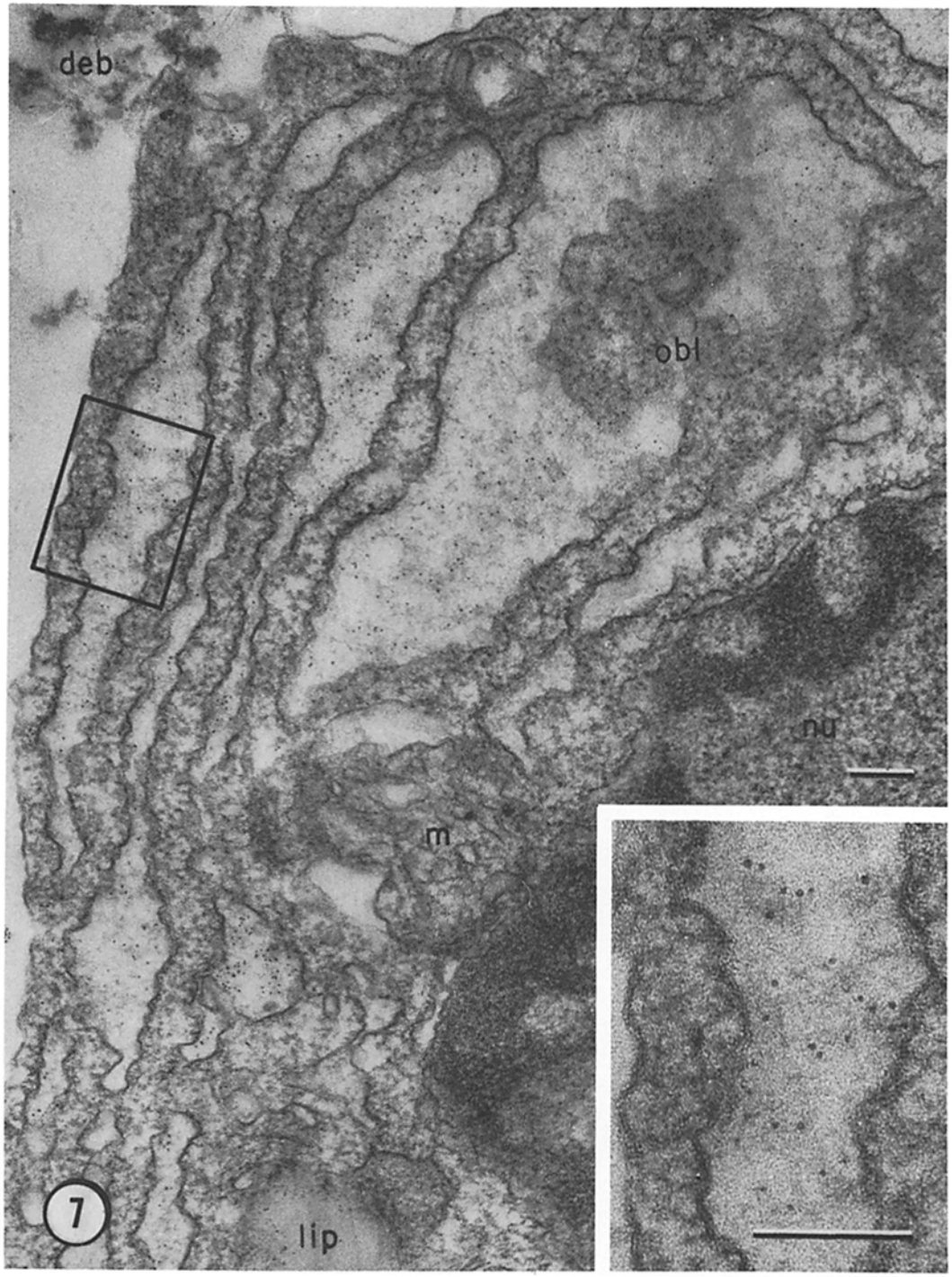


FIGURE 7. The intracisternal contents of tendon fibroblasts labeled with antipro-α1 are shown to good advantage in this electron micrograph of a section stained with lead citrate. The reticulum is sectioned obliquely at *obl*. Extracellular debris (*deb*) may be labeled, probably due to the presence of antigen extruded from damaged cells. *m*, mitochondrion; *nu*, nucleus; *lip*, lipid with uranium precipitates from en bloc staining. × 90,000. The inset shows the area in the rectangle at high magnification. × 230,000.

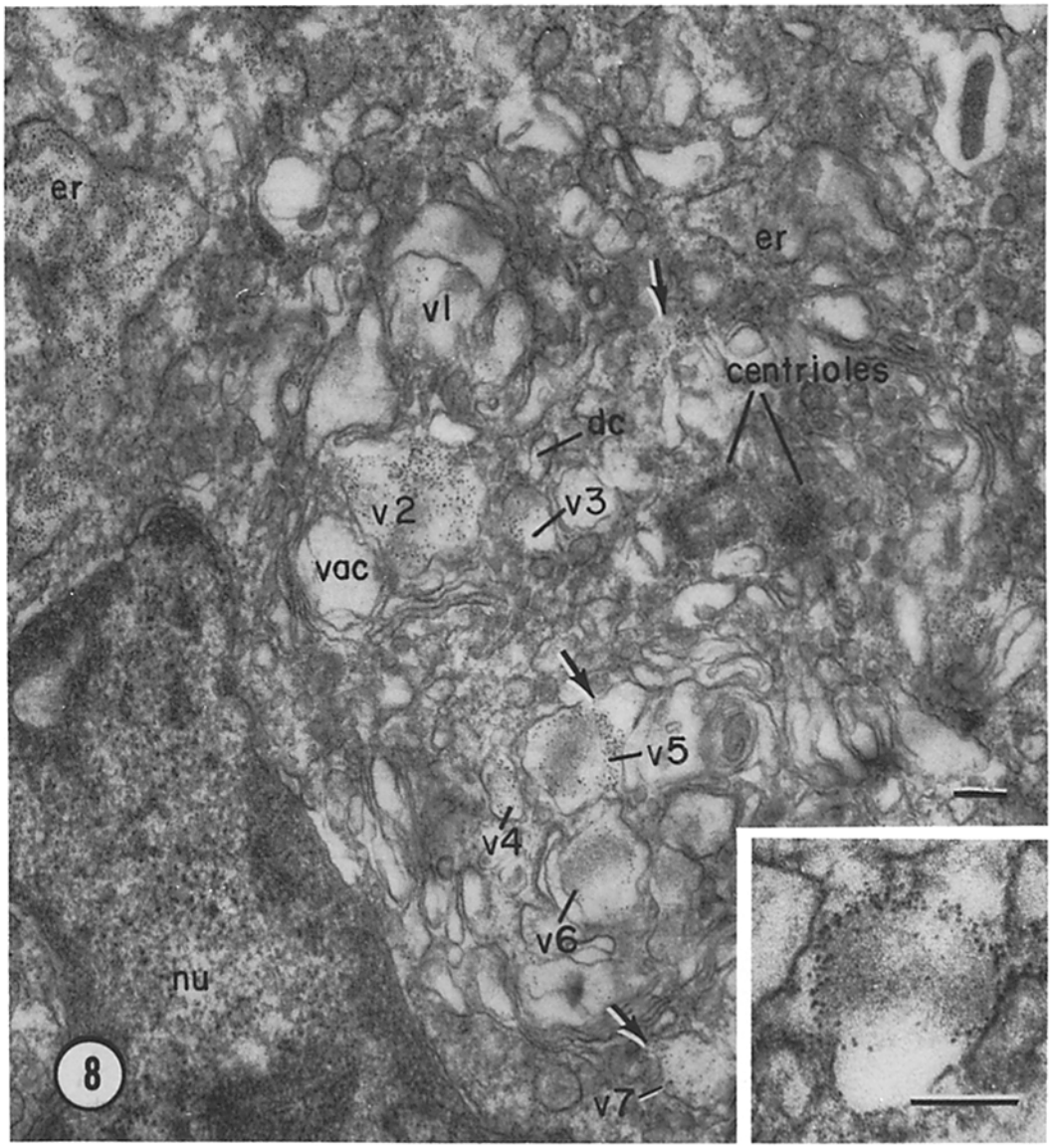


FIGURE 8. The Golgi vacuoles (*v*) of tendon fibroblasts labeled with antibody (antipro-α1 in this case) when the bounding membranes are broken. Many vacuoles (*v 1–7*) are labeled in this micrograph. Electron-dense contents may be completely (*vac*) or partially (*v 1*) lost. Arrows indicate breaks in Golgi vacuoles. *nu*, nucleus; *dc*, dilated cisterna. × 70,000. The inset shows a secretory granule, at higher magnification, from another fibroblast in the same preparation. Lead citrate stain. × 140,000.

apparently responds according to its own shape and mechanical stability. Thus, the extended cisternae of the endoplasmic reticulum appear to break rather easily (23, 24), allowing entry by diffusion of the ferritin-conjugated antibody and resulting in a high frequency of images of well-preserved portions of cisternae (remote from the breakage point) containing large concentrations of antibody.

The Golgi apparatus, consisting of smaller cisternae and vesicles, presents fewer opportunities for breakage. The Golgi elements require more force for disruption, as evidenced by the fact that fragmented Golgi vacuoles and vesicles are usually

found in cells where the endoplasmic reticulum is also largely fragmented. On the other hand, when Golgi vacuoles are broken, their empty membranes tend to form myelin figures. Since the homogenization produces a distribution between heavily damaged and intact, unlabeled Golgi complexes it is possible to find intermediate images that demonstrate both recognizable ultrastructure and penetration of ferritin-conjugated antibody. Thus, only recognizable Golgi regions containing antibody can be considered meaningful.

Of the recognizable Golgi elements that contained antibody, the large vacuoles were the best preserved, and in tendon fibroblasts their contents were condensed and tended to remain intact although their bounding membranes were damaged.

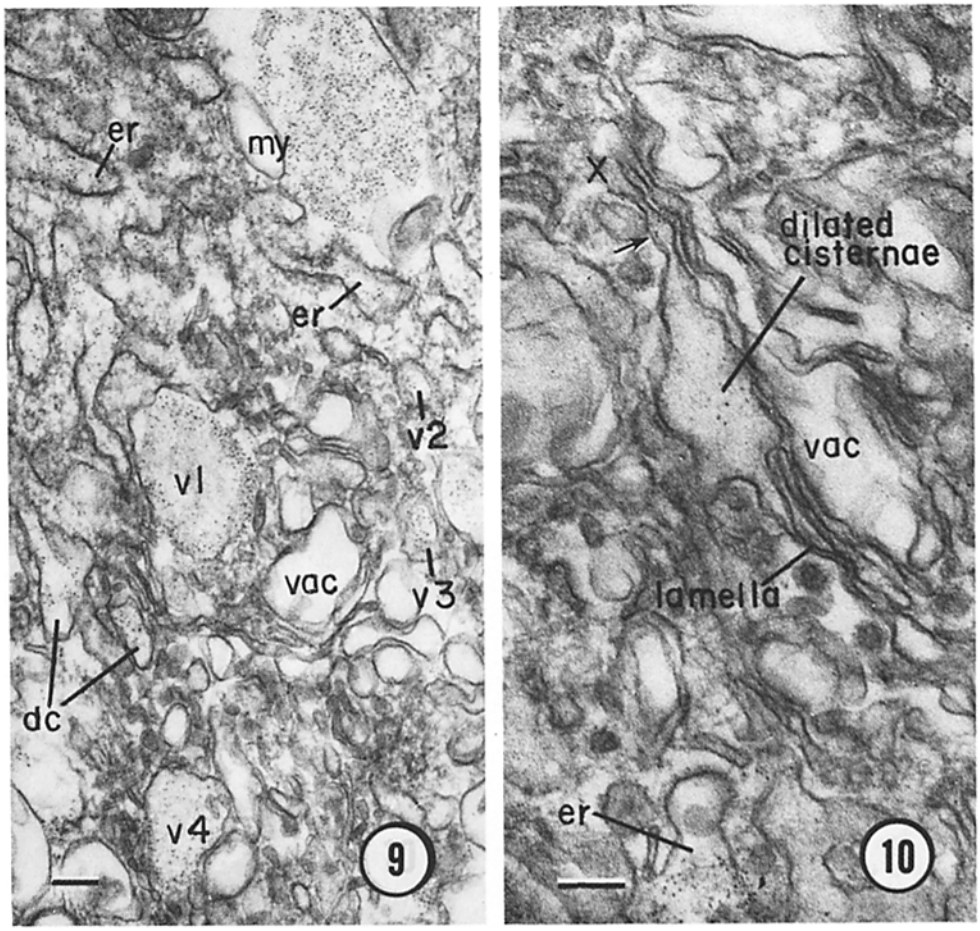


FIGURE 9. Antiprocollagen B labels Golgi vacuoles and vesicles (*v*) and endoplasmic reticulum (*er*) in the same manner as do other antiprocollagens. Vacuole 1 contains a secretory granule similar to the one shown in Fig. 8, *inset*. Two large vesicles (*v2*, *v3*) and dilated cisternae (*dc*) are labeled. Vacuole *v4* may be either a tangential section of a large vacuole or a transition size between *v1* and *v2*. An empty vacuole appears at *vac*. In the upper right, the membrane of the reticulum has been damaged and reappears in part as a myelin figure (*my*) after releasing its contents. Lead citrate stain. × 70,000.

FIGURE 10. The Golgi zone of a corneal fibroblast treated with anti-T3 illustrated here contains only a few ferritin-labeled antibodies in a dilated cisterna. Negative results are difficult to interpret because of the necessity of breaking membranes to allow the antibody to enter each individual Golgi element. Breaks in the membrane may allow the contents of a vacuole or cisterna to leak out, so that the cavity seems lacking in electron density (*vac*). However, the small arrow points to ferritin particles in a lamella; a membrane flaw that may account for this labeling is located at *x*. *er*, endoplasmic reticulum. Lead citrate stain. × 90,000.

Thus, it seems reasonable to refer to these 200-nm diameter units as secretory vacuoles or granules. It is possible that they represent the compartment in which procollagen is transported to the cell surface (11, 12, 24, 26). It is not possible either with this technique or with autoradiography to rule out direct transport of some procollagen from the endoplasmic reticulum to the exterior via small vesicles (27). The antibody-labeled small vesicles observed in well-fragmented Golgi zones, however, may have been transporting procollagen to the Golgi zone.

Neither the corneal nor the tendon fibroblast contains the dense oblong secretory granules observed in corneal epithelium (12, 13, 31) and in odontoblasts (34). It could be argued that the oblong "condensation" granules formed and were discharged so rapidly that they were not observed by our method. However, it seems more likely that different kinds of collagen-secreting cells produce different types of secretory granules, depending on the nature of the noncollagenous products also secreted by these cells. In well-fragmented Golgi zones, we observed antibody labeling of essentially the whole organelle, including the rounded "procollagen granules" alluded to above. We should have seen the cigar-shaped "secretory granules" (31) if they were present, even in small numbers.

The immunocytochemical method used here has the advantage of specificity, but lacks the ability to provide quantitative (or kinetic) data. Thus it is not possible with this technique to determine whether all or only part of the collagen secreted by the cells utilizes the Golgi complex. Characteristics of the method also make negative results inconclusive. The method's greatest potential lies in the identification of proteins synthesized, secreted, or taken up by a given cell, and the distribution of these protein antigens within the cell.

We are grateful to Eva Marie Click for assistance with the immunoassays and to Kathy Kiehnau and Willie Mae Young for assistance in preparation of specimens for electron microscopy.

This investigation was supported by National Institutes of Health grants AM 11248, DE 02600, AM 13970, HD 00143, and AM 16516. Dr. Nist is the recipient of a National Institutes of Health postdoctoral fellowship grant no. HL 54489. Paul Bornstein was the recipient of research career development award K4-AM 42582 from the United States Public Health Service.

Received for publication 28 June 1974, and in revised form 30 December 1974.

BIBLIOGRAPHY

1. AINSWORTH, S. K., and M. J. KARNOVSKY. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* **20**:225–229.
2. BORNSTEIN, P. 1974. The biosynthesis of collagen. *Annu. Rev. Biochem.* **43**:567–603.
3. BORNSTEIN, P., and H. P. EHRLICH. 1973. The intracellular translocation and secretion of collagen. *In* Biology of Fibroblast. E. Kulonen and J. Pikkarainen, editors. Academic Press, Inc., London. 321–338.
4. CONRAD, G. W. 1970. Collagen and mucopolysaccharide biosynthesis in mass cultures and clones of chick corneal fibroblasts in vitro. *Dev. Biol.* **21**:611–635.
5. COOPER, G. W., and D. J. PROCKOP. 1968. Intracellular accumulation of protocollagen and extrusion of collagen by embryonic cartilage cells. *J. Cell Biol.* **38**:523–537.
6. CUATRECASAS, P. 1970. Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. Biol. Chem.* **245**:3059–3065.
7. DEHM, P., B. R. OLSEN, and D. J. PROCKOP. 1974. Antibodies to chick tendon procollagen. Affinity purification with the isolated disulfide-linked NH_2-terminal extensions and reactivity with a component in embryonic serum. *Eur. J. Biochem.* **46**:107–116.
8. DEHM, P., and D. J. PROCKOP. 1971. Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon. *Biochim. Biophys. Acta.* **240**:358–369.
9. EDELMAN, G. M., and J. J. MARCHALONIS. 1967. Methods used in studies of the structure of immunoglobulins. *In* Methods of Immunology and Immunochemistry. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. **1**:405–424.
10. FAHEY, J. L. 1967. Chromatographic separation of immunoglobulins. *In* Methods in Immunology and Immunochemistry. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. **1**:321–332.
11. FRANK, R. M. 1970. Etude autoradiographique de la dentinogenèse en microscopie electronique à l'aide de la proline tritée chez le chat. *Arch. Oral Biol.* **15**:583–596.
12. HAY, E. D., and J. W. DODSON. 1973. Secretion of collagen by corneal epithelium. I. Morphology of the collagenous products prepared by isolated epithelia grown on frozen-killed lens. *J. Cell Biol.* **57**:190–213.
13. HAY, E. D., and J. P. REVEL. 1969. Fine structure of the developing avian cornea. Monographs in Developmental Biology. A. Wolski and P. S. Chen, editors. S. Karger AG., Basel. Vol. I.
14. JAMIESON, J. D., and G. E. PALADE. 1967. Intracellu-

lar transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* **34**:577–615.

15. KRAEHENBUHL, J. P., and J. D. JAMIESON. 1972. Solid phase conjugation of ferritin to Fab-fragments of immunoglobulin G for use in antigen localization on thin sections. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1771–1775.
16. LAYMAN, D., and R. ROSS. 1973. The production and secretion of procollagen peptidase by human fibroblasts in culture. *Arch. Biochem. Biophys.* **157**:451–456.
17. LEDUC, E. H., G. B. SCOTT, and S. AVRAMEAS. 1969. Ultrastructural localization of intracellular immune globulins in plasma cells and lymphoblasts by enzyme-labeled antibodies. *J. Histochem. Cytochem.* **17**:211–224.
18. MARCHALONIS, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113**:299–305.
19. MARTINEZ-HERNANDEZ, A., G. B. PIERCE, and P. K. NAKANE. 1973. Intracellular localization of basement membrane antigen in normal and neoplastic parietal yolk sac cells. *J. Cell Biol.* **59**(2, Pt. 2):217 a. (Abstr.).
20. MCLEAN, J. D., and S. J. SINGER. 1970. A general method for the specific staining of intracellular antigens with ferritin-antibody conjugates. *Proc. Natl. Acad. Sci. U. S. A.* **65**:122–128.
21. MONSON, J. M., and P. BORNSTEIN. 1973. Identification of a disulfide-linked procollagen as the biosynthetic precursor of chick bone collagen. *Proc. Natl. Acad. Sci. U. S. A.* **70**:3521–3525.
22. NEUTRA, M., and C. P. LEBLOND. 1966. Synthesis of the carbohydrate of mucus in the Golgi complex as shown by electron microscope radioautography of goblet cells from rat injected with glucose-H_3. *J. Cell Biol.* **30**:119–136.
23. OLSEN, B. R., R. A. BERG, Y. KISHIDA, and D. J. PROCKOP. 1973. Collagen synthesis: Localization of prolyl hydroxylase in tendon cells detected with ferritin-labelled antibodies. *Science (Wash. D. C.).* **182**:825–827.
24. OLSEN, B. R., and D. J. PROCKOP. 1974. Ferritin-conjugated antibodies used for labeling of organelles involved in the cellular synthesis and transport of procollagen. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2033–2037.
25. REVEL, J. P. 1970. Role of the Golgi apparatus of cartilage cells in the elaboration of matrix glycosaminoglycans. *In* Chemistry and Molecular Biology of the Intercellular Matrix. E. A. Balazs, editor. Academic Press, Inc., New York. 1485–1502.
26. REVEL, J. P., and E. D. HAY. 1963. An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage. *Z. Zellforsch. Mikrosk. Anat.* **61**:110–144.
27. ROSS, R., and E. P. BENDITT. 1965. Wound healing and collagen formation. V. Quantitative electron microscope autoradiographic observations of proline-^{3}H utilization by fibroblasts. *J. Cell Biol.* **27**:83–106.
28. SALPETER, M. M. 1968. H^3-proline incorporation into cartilage: electron microscopic autoradiographic observations. *J. Morphol.* **124**:387–421.
29. SCHOFIELD, J. D., and D. J. PROCKOP. 1973. Procollagen—a precursor form of collagen. *Clin. Orthop. Relat. Res.* **97**:175–195.
30. SMITH, B. D., P. H. BYERS, and G. R. MARTIN. 1972. Production of procollagen by human fibroblasts in culture. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3260–3262.
31. TRELSTAD, R. L. 1971. Vacuoles in the embryonic chick corneal epithelium, an epithelium which produces collagen. *J. Cell Biol.* **48**:689–694.
32. VON DER MARK, K., and P. BORNSTEIN. 1973. Characterization of the proα1 chain of procollagen. Isolation of a sequence unique to the precursor chain of procollagen. *J. Biol. Chem.* **248**:2285–2289.
33. VON DER MARK, K., E. M. CLICK, and P. BORNSTEIN. 1973. The immunology of procollagen. I. Development of antibodies to determinants unique to the proα1 chain. *Arch. Biochim. Biophys.* **156**:356–364.
34. WEINSTOCK, M., and C. P. LEBLOND. 1974. Synthesis, migration, and release of precursor collagen by odontoblasts as visualized by autoradiography after ^{3}H-proline administration. *J. Cell Biol.* **60**:92–127.