Impaired Conversion of Procollagen to Collagen by Fibroblasts and Bone Treated with Tunicamycin, an Inhibitor of Protein Glycosylation*

(Received for publication, August 19, 1976)

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Tunicamycin, an inhibitor of lipid carrier-dependent protein glycosylation, was used in studies of procollagen synthesis, secretion, and proteolytic modification by chick cranial bones in organ culture and by chick tendon fibroblasts in tissue culture. Tunicamycin inhibited the incorporation of D-\(^{12-3}H\)mannose into procollagen by greater than 90% whereas general protein synthesis and collagen synthesis were decreased by only 10 to 20%. The procollagen synthesized in the presence of tunicamycin was secreted normally and its immunological characteristics, as detected by an antiserum to the intact protein, were unchanged. However, tunicamycin caused an accumulation of biosynthetic intermediates containing disulfide-bonded COOH-terminal extensions in both cell and bone culture. Cleavage of NH\(_2\)-terminal extensions was not detectably impaired. These findings provide additional support for the involvement of more than one enzyme in the limited proteolytic conversion of procollagen to collagen.

The identification of a biosynthetic precursor of collagen, procollagen (see Refs. 2 and 3 for reviews), has been followed by studies directed toward an understanding of the molecular structure of the precursor, its function, and the process by which it is converted to collagen. Intact type I procollagen contains nontriple helical domains at both the NH\(_2\)- and COOH-terminal ends of the molecule while interchain disulfide bonds, characteristic of the precursor, are confined to the larger COOH-terminal domain (4-6). In addition, procollagen contains carbohydrate moieties, at least some of which have been shown to be located in the COOH-terminal nontriple helical domain (7-9). The biological functions of the nontriple helical domains are not well understood. Recent data support the postulate (10) that the COOH-terminal end serves to initiate chain association and subsequent triple helix formation (11). Previously, studies of the rate of polymerization and cross-link formation using dermatosparitic calf skin collagen had indicated that these processes were retarded unless NH\(_2\)-terminal extensions were excised (12).

Since peptide sequences from both the NH\(_2\)- and COOH-terminal regions must be removed during conversion of procollagen to collagen, it seems likely that more than one enzymatic activity is involved. Data from several laboratories have provided experimental evidence for stepwise conversion and for intermediates in the conversion process (6, 13-15). During conversion, the nontriple helical extensions are excised from the collagenous portion of procollagen to yield an intact COOH-terminal carbohydrate-containing fragment (7, 15). The function of the carbohydrate in this nontriple helical domain is not understood, but, in view of studies with other proteins, it appeared that it could be important for secretion and for the regulation of conversion of procollagen to collagen. To examine these possibilities we have used tunicamycin, an inhibitor of lipid carrier-dependent protein glycosylation (16-18), to produce nonglycosylated collagen precursors and to study its effects on the synthesis, secretion, and enzymatic conversion of procollagen to collagen in chick cranial bones and in chick tendon fibroblasts in culture. Although synthesis and secretion of procollagen were only minimally affected by tunicamycin, the conversion of procollagen to collagen was impaired, and this impairment was due to failure of cleavage of the COOH-terminal domain. Since secretion of some other proteins appeared to be markedly affected in this study there may be both glycosylation-dependent and -independent secretory pathways for extracellular proteins.

EXPERIMENTAL PROCEDURES

**Materials**—Powdered DMEM, fetal calf serum, penicillin, and streptomycin were purchased from Grand Island Biological Co.; crude and purified (CLSMA) collagenases were from Worthington Biochemical Corp.; trypsin (1.590) from ICN Pharmaceutical, Inc.; EDTA disodium salt (puriss) from Fluka A. G.; Hepes from Sigma Chemical Co.; and butyl p-hydroxybenzoate from Matheson, Coleman and Bell. Tunicamycin, batches T-11-05 and T-12-06, were a gift from Dr. Gakuzo Tamura, Department of Agricultural Chemistry, University of Tokyo. The following radiochemicals were purchased from New England Nuclear: L-\(^{[2,3-3}H\)proline (35 Ci/mmol), \(^{[2-3}H\)leucine (36.02 Ci/mmol), \(^{[2-3}H\)tryptophan (7.7 Ci/mmol), L-\(^{[2-\text{nuclease}}\)cystine (36.02 Ci/mmol), L-\(^{[2-3}H\)glucosamine hydrochloride (10 Ci/mmol), D-\(^{[2-3}H\)mannose (4.4 mCi/mmol) and D-\(^{[2-3}H\)mannose (2 mCi/mmol) were purchased from Amersham/Searle Co. Plastic tissue culture dishes (100 mm diameter) were purchased from Falcon Plastics.

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*This work was supported by National Institutes of Health Grants DE-02600 and AM-11248. A preliminary report of part of this work was published elsewhere (1).*
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**Tissue and Cell Culture** - Chick embryos (17 days old) were obtained from a local hatchery. Chick leg tendon fibroblasts were isolated as previously described (10) with the modification that minced tendons were digested for 60 min at 37° with a 1:1 mixture of trypsin (0.25%) and EDTA (0.02%) solutions (20). Primary monolayer cultures were obtained by seeding 5 × 10^6 freshly dissociated cells per plate in DMEM supplemented with 27 mM NaHCO₃, 15 mM HEPES (pH 7.4, penicillin (100 units/ml), streptomycin sulfate (100 mg/ml), butyryl/ hydroxybenzoate (0.2 mg/liter), and 10% (v/v) fetal calf serum. This medium is referred to as culture medium. The cultures were maintained in a humidified incubator at 5% CO₂, 95% air, and were used for experiments 3 to 4 days after seeding. Cranial bones were removed from 17-day-old chick embryos and placed directly into serum-free culture medium (0.5 ml/calvarium) (21).

**Incorporation of Amino Acids and Sugars and Measurements of Collagen Synthesis** - Preliminary experiments indicated that a 6-h preincubation period was needed for maximal effect of tunicamycin on the incorporation of sugars into glycoproteins (22). Chick embryo cranial bones or monolayers of chick embryo tendon fibroblasts were washed twice with DMEM to remove excess serum, and preincubated for 6 h in the presence or absence of tunicamycin (5 μg/ml) in labeling medium which was composed of serum-free culture medium supplemented with sodium ascorbate, glutamine, and β-amino-propionitrile as previously described (21). Subsequently, the cells or bones were washed again and incubated for 2 h with the radioactive amino acid or sugar in the presence or absence of tunicamycin. Bones were washed once, and the volume of 1 ml labeled amino acid or sugar was added at a concentration of 2 μCi/ml in cell cultures and 5 μCi/ml in bone cultures. When labeling was done with a radioactive amino acid, that amino acid was omitted from the labeling medium; when a radioactive sugar was used, the glucose concentration in the labeling medium was reduced to 50 mg/liter and the medium was supplemented with 1.1 g/liter of sodium pyruvate as an energy source (23). At the end of the labeling period protein synthesis was stopped by rapidly cooling the plates or bottles to 0°. All subsequent treatments were performed at 0°, and the medium and supernatant fractions of tunicamycin, and the medium and cell layer were washed twice with DMEM to remove excess serum, and preincubated for 6 h in the presence or absence of 0.5 to 5.0 μg of tunicamycin/ml of culture medium. The drug was prepared and purified by chromatography on DEAE-cellulose (24) of the Lowry method using bovine serum albumin as a standard. Proteins of both the medium and the tissue were precipitated with a 10% trichloroacetic acid containing 10 mM NaCl, 0.05 M Tris/HCl (pH 7.5), 5 mM CaCl₂, and made 10 mM in N-ethylmaleimide. The dialyzed sample was incubated for 2 h at 37° with 50 μM of purified collagenase (25). The reaction was terminated by addition of glacial acetic acid and heating to 65° for 10 min; aliquots were then removed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analysed Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis - Gel electrophoresis was carried out on 5% acrylamide gels (13 × 0.6 cm) for procollagen and procollagen-derived intermediates (13) and 7.5% acrylamide gels (13 × 0.6 cm) for the procollagen-derived COOH-terminal peptides from culture medium of bones (7). The gel electrophoresis system was used as described by Goldberg et al. (27) except that an equimolar concentration of N,N'-diallyltarlardiamine was substituted for methylenebisacrylamide as a cross-linking agent (28). Dansylated collagen α chains, β₁ and components, and α₁(1-110)-terminal peptide from culture medium of bones (7).

**RESULTS**

**Effect of Tunicamycin on Protein Synthesis** - The effect of tunicamycin was tested in chick embryo cranial bones and chick embryo tendon fibroblasts. In both systems the incorporation of amino acids into macromolecules was decreased by only 10 to 20% in the presence of tunicamycin (Table I). Incorporation of D-[6-3H]glucosamine and D-[1-3H]mannose, which retain the label despite metabolic conversions, was inhibited by about 80% in tendon fibroblasts. Incorporation of D-[2-3H]mannose, which loses the label during enzymatic conversion to fructose and to all other sugars except fucose, was inhibited almost completely (>95%) in the presence of tunicamycin (Table I). Thus, in agreement with previous work (22), tunicamycin inhibits the incorporation of sugars into glycoproteins without markedly inhibiting protein synthesis.

**Effect of Tunicamycin on Collagen Synthesis and Secretion** - The effect of tunicamycin on the synthesis and secretion of collagenous proteins by chick embryo leg tendon fibroblasts was tested as follows. Following a preincubation period of 6 h in the presence or absence of 0.5 to 5.0 μg of tunicamycin/ml of labeling medium, cells were further incubated for 24 h with [1-3H]proline in medium containing these different concentrations of tunicamycin, and the medium and cell layer were analyzed for collagenous protein at the end of this period. Tunicamycin inhibited [1-3H]proline incorporation into total protein and into collagenous proteins by about 20%, both in the medium and the cell fraction, but the distribution between medium and cell layer was unchanged (Table II). The drug therefore does not specifically inhibit secretion of procollagen.

**Long Term Double Labeling of Fibroblasts** - Although synthesis and secretion of procollagen were not significantly al-
B) and tunicamycin-treated fibroblasts (Fig. 1, C and D) were intermediates also migrated between the above Dns markers from that gel electrophoresis. When the media of control (Fig. 1, presence or absence of 2 pg/ml of tunicamycin. Proteins from collagenase-sensitive. After reduction, procollagen and its in-

precursor. To examine this possibility, fibroblasts were la-
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tinated by tunicamycin, the absence of the precursor-specific carbohydrate might affect the subsequent metabolic fate of the precursor. To examine this possibility, fibroblasts were labeled for 24 h with [14C]proline and n-[2-3H]mannose in the presence or absence of 2 \( \mu \)g/ml of tunicamycin. Proteins from the medium were isolated and separated by polyacrylamide gel electrophoresis. When the media of control (Fig. 1, A and B) and tunicamycin-treated fibroblasts (Fig. 1, C and D) were examined a marked inhibition of incorporation of radioactive mannose into proteins was observed. The only fraction in which such inhibition was not observed was the highest mole-
cular weight component that appeared near the top of the gel. This component probably contained protein-polysaccharides.

Procollagen, disulfide-bonded intermediates which migrated 15 to 25 mm from the top of unreduced gels, and \( \alpha \) chains which migrated between Dns-\( \beta_{1} \), and Dns-\( \alpha_{1} \) markers were collagenase-sensitive. After reduction, procollagen and its intermediates also migrated between the above Dns markers (Fig. 1, B and D).

In medium from tunicamycin-treated cells (Fig. 1, C and D) the distribution of the [14C]proline label differed markedly from that of control cells. The ratio, calculated from 6 experiments, of [14C]radioactivity in procollagen plus intermediates to [14C]radioactivity in \( \alpha \) chains was 0.93 \pm 0.2 for control cultures and 2.52 \pm 0.5 for tunicamycin-treated cultures. The accumu-
lation of procollagen and intermediates in the presence of tunicamycin indicated an inhibition of conversion of procolla-
gen to collagen. It was interesting to note that a collagenase-
resistant proline- and mannose-labeled peak, corresponding in position of migration to the elastic fiber microfibrillar protein isolated by Muir et al. (35) was absent from the tunicamycin-treated medium. This glycoprotein, in the reduced form, is indicated by the black bar in Fig. 1B.

When the collagenous proteins in the cell layer were ana-
alyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis most of the [14C]proline label appeared in \( \alpha_{1} \) and \( \alpha_{2} \) chains. There was no accumulation of intracellular procolla-
gen in either control or tunicamycin-treated cultures (data not shown).

To further characterize the procollagen produced in the presence of tunicamycin, medium from fibroblasts labeled with [14C]proline and [14H]mannose was precipitated with antibi-
dodies directed against determinants in COOH- and NH2-
terminal extensions of procollagen. Approximately 98% of the total [14C]proline in the control medium and 94% of the total [14C]proline in the tunicamycin-treated medium was precipitated by the antiserum (Fig. 2A). The fraction precipitated from the medium of bone organ cultures labeled with [3H]tryptophan was also the same for control and tunicamycin-
treated cultures (Fig. 2B). These findings indicate that the antigenicity of procollagen lacking carbohydrate was unchanged, as detected by the antisera used, and suggest that carbohydrate side chains may not be the major antigenic determinants in procollagen. It should be noted that immune precipitation of collagenous proteins does not distinguish be-
 tween procollagen and the several intermediates in conversion of procollagen to collagen since all of these proteins contain COOH-terminal or NH2-terminal extensions of procollagen. After denaturation and electrophoresis, a different spectrum of in-
termediates may be revealed by the different relative propor-
tions of \( \alpha \) chains and disulfide-bonded forms, as seen in Fig. 1, A and C, even though the extent of immune precipitation of the two preparations was the same.

The [14C]proline:[3H]mannose ratio in the immune precipi-
tates was approximately 1.9:1 for the control fibroblast me-
tations.
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FIG. 1. Dodecyl sulfate-acrylamide gel electrophoresis of proteins labeled for 24 h with \(^{14}C\)proline and \(^{2-3}H\)mannose and precipitated from medium of cultured chick embryo tendon fibroblasts. A, control culture prior to reduction; B, control culture after reduction; C, tunicamycin-treated culture prior to reduction; D, tunicamycin-treated culture after reduction. The arrows indicate the position of migration of dansylated \(\beta_1\), \(\alpha_1\) and \(\alpha_1\)-CB7 components, and the dye marker. The black bar in B indicates the position of migration of a collagenase-resistant glycoprotein.

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FIG. 2. Immune precipitation curves of: A, \(^{14}C\)proline-labeled medium from tendon fibroblast culture; B, \(^{3}H\)tryptophan-labeled medium from bone organ culture. - - - , control medium; O - - - O, tunicamycin-treated medium. Anti-procollagen serum was diluted 1:50. Points represent the average of triplicate assays using about 5,000 cpm of antigen.

Pulse-Chase Experiments in Cultured Fibroblasts—To obtain a better understanding of the mechanism of the inhibitory effect in conversion of procollagen to collagen observed with tunicamycin, a pulse-chase experiment was performed with fibroblasts in culture. A pulse period of 45 min with \(^{3}H\)proline was followed by an 18-h chase with medium containing an excess of unlabeled proline. The collagenous proteins were separated by slab gel sodium dodecyl sulfate-polyacrylamide electrophoresis under reducing conditions and the \(^3\)H-labeled protein bands visualized by fluorescent autoradiography (Fig. 3). At the end of the pulse, \(\alpha_2\) chains were present in both tunicamycin-treated and control medium.

FIG. 3. Composite fluorescent autoradiograms of a \(^{3}H\)proline-labeled protein isolated from medium of cultured fibroblasts and electrophoresed on dodecyl sulfate-acrylamide slab gels under reducing conditions. Cultures were pulsed for 45 min with \(^{3}H\)proline and chased for 3, 6, and 18 h with excess unlabeled proline. Approximately 5,000 cpm were applied to each slot of the gel. The arrows indicate the portion of migration of dansylated \(\alpha_1\), \(\alpha_1\)-CB7, and the dye marker.
However, the course of conversion was considerably slower in tunicamycin-treated cultures. At the end of an 18-h chase, only proa and pα chains\(^2\) were present in tunicamycin-treated cultures whereas α chains made up a prominent portion of the collagenous proteins in control cultures both in the medium (Fig. 3) and in the cell layer (data not shown). These findings indicate that the proteolysis of the COOH-terminal extension was markedly inhibited in the tunicamycin-treated cultures.

**Long Term Labeling of Bones**—The course of procollagen to collagen conversion has been well studied in chick cranial bones in culture. Conversion can occur within a physiological setting (in contrast to cells in culture) and intermediates in the process have been identified (6, 13). Chick embryo cranial bones were incubated as described under "Experimental Procedures" and the proteins in the culture medium analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). The medium from control cultures (Fig. 4, A and B) contained two major reducible peaks that were labeled with cystine and mannose. A high molecular weight, collagenase-sensitive peak was identified as procollagen; a second peak migrated ahead of Dns-cul in the nonreduced gel and was identified as COOH-terminal procollagen-derived peptide (7). This peptide migrated more rapidly than the Dns-al-CB7 marker in the reduced gel and contained approximately 10% of the \[^{35}S\]cystine radioactivity. In addition to these two reducible peaks there were other nonreducible peaks which were not identified.

In the medium from tunicamycin-treated bones (Fig. 4, C and D) procollagen and the COOH-terminal procollagen-derived peptide lacked the mannose label almost completely. The medium peptide peak appeared smaller (presumably due to lack of carbohydrate) and migrated further into the gel. In three experiments the total \[^{35}S\]cystine radioactivity found in the medium peptide peak of tunicamycin-treated bones was 50% of that found in the medium peptide of control bones, i.e. approximately 5% of the total radioactivity. When the media were precipitated with antiprocollagen serum the ratio of \[^{35}S\]cystine: \[^{3}H\]mannose was 0.3:1 in control medium and 8.7:1 in tunicamycin-treated medium.

To further characterize the glycosylated and nonglycosylated COOH-terminal peptides from procollagen, bones were incubated with \[^{3}H\]tryptophan for 2 h in the presence or absence of tunicamycin, the media were collected, and proteins precipitated by trichloroacetic acid. The \[^{3}H\]tryptophan-labeled COOH-terminal peptide was further purified by chromatography on DEAE-cellulose (7). Peptides from control and tunicamycin-treated bones were then analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels at acrylamide concentrations of 7.5, 10, 12.5, or 15% to determine an asymptotic value for migration distance relative to the observed migration of Dns-al-CB7, a nonglycosylated collagenous peptide of approximately 25,000 daltons (Fig. 5). The reduced procollagen-derived medium peptide synthesized by bones in the presence of tunicamycin showed the same migration as Dns-al-CB7 at all gel concentrations, whereas the reduced peptide, synthesized under control conditions, showed a slower migration typical of proteins with high sugar content (35). The asymptotic value for the molecular weight of the reduced glycosylated peptide relative to three noncollagen protein standards was 39,500 while that for the reduced nonglycosylated peptide was 37,500.

**Pulse-Chase Experiments in Bones**—Pulse-chase experiments were performed in bones to follow the conversion process and to further clarify the nature of the tunicamycin effect. For these experiments cranial bones were preincubated for 6 h and then incubated with \[^{3}H\]proline for 18 min in the presence or absence of tunicamycin, transferred to medium with an excess of unlabeled proline, and incubated further for 30 or 90 min. The collagenous proteins were then extracted and the...
samples analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. After an 18-min pulse, virtually all the radioactivity in nonreduced control and tunicamycin-treated samples was found in a single, high molecular weight peak with a molecular weight in excess of 400,000 (Fig. 6, A and B). After reduction, two bands with apparent molecular weights of approximately 150,000 were obtained (Fig. 6, A and B) and identified as proα1 and proα2 (13). After a 90-min chase (Fig. 6, C and D), there was a decrease in the relative amount of intact procollagen accompanied by a corresponding increase of two lower molecular weight, nonreducible components whose mobilities coincided with those of α1 and α2 chains. There was a marked difference between control bones and tunicamycin-treated bones. In the control bones (Fig. 6C), almost all the procollagen was converted to collagen and only 10% of the radioactivity was found in high molecular weight intermediates. In contrast, 55% of the radioactivity remained in high molecular weight intermediates in bones treated with tunicamycin (Fig. 6D). These results are in agreement with those previously obtained with fibroblasts in culture (Fig. 1).

To demonstrate the step at which inhibition of conversion occurred, a shorter chase period of 30 min was used. After an 18-min pulse, both control and tunicamycin-treated bones contained proα1 and proα2 chains (Fig. 7). In control samples, after a 30-min chase (Fig. 7C), all the potential intermediates in the conversion pathway, pαα and pαα chains as well as proα and αα chains, were present (6, 13, 15). The predominant peaks were the α1 and α2 chains as expected in the normal conversion process. In the tunicamycin-treated bones (Fig. 7D), there were striking differences. Smaller amounts of α1 and α2 chains and an accumulation of pαα1 chains were seen, indicating an impairment in the activity of the COOH-terminal cleavage enzyme in the presence of tunicamycin. The accumulation of pαα1 chains appeared more pronounced than that of pαα2. No corresponding increase in pαα2 was observed suggesting that cleavage of NH2-terminal extensions was not impaired by tunicamycin.

Consistent results were obtained by examination of samples prior to reduction. Thus, after an 18-min pulse and 30-min chase, pαcollagen was found in similar amounts in control and tunicamycin-treated bones (data not shown). This finding again suggests that the activity of the NH2-terminal procollagen protease was not markedly affected by tunicamycin.

**DISCUSSION**

The data presented in this paper clearly indicate that in the presence of tunicamycin the conversion of procollagen to collagen is inhibited. The limited proteolysis of procollagen is a complex process that involves the cleavage of at least 6 peptide...
acetylglucosaminyl pyrophosphate from UDP-N-acetylglucosamin-specifically inhibits the formation of polyisoprenyl N-terminus with calf liver microsomes (16), chick embryo microsomes (17), and yeast protoplasts (18) have shown that tunicamycin, a glycoprotein that is synthesized as an inactive zymogen and normally activated extracellularly. In this study, procollagen synthesized in the presence of tunicamycin was secreted normally by fibroblasts, evidence against linkage of oligosaccharides to seryl or threonyl residues was provided, suggesting, by exclusion, linkage to asparaginyl side chains. The hypothesis that sugar moieties are necessary substituents of proteins designed for export from the cell (42) has been questioned (43). In this study, procollagen synthesized in the presence of tunicamycin was secreted normally by fibroblasts in culture and probably by cranial bone. Such procollagen can be assumed to lack oligosaccharides linked by asparagine-N-acetylgalactosamine bonds. Tunicamycin probably does not inhibit the addition of galactosyl and glucosyl moieties to hydroxylsyl residues in the triple helical region of the protein. Indeed, in most glycoproteins in which carbohydrate is attached to asparagine, N-acetylgalactosamine linked N-glycosidically, is followed by mannos (40, 41). Clark and Kefalydis (9) have recently reported the presence of glucosamine, galactosamine, and mannos in type I procollagen isolated from chick tendon fibrils. Evidence against linkage of oligosaccharides to seryl or threonyl residues was provided, suggesting, by exclusion, linkage to asparaginyl side chains. Several studies (38, 39) have indicated that glycoprotein precursors of viral structural proteins failed to be converted properly when glycosylation of the protein was inhibited by deoxyglucose or glucosamine; in the case of influenza virus hemagglutinin, the presence of heterogenous cleavage products was attributed to lack of substrate specificity caused by the lack of sugar side chains (39). In preliminary studies with enzyme extracted from fibroblast medium (15), we found that tunicamycin was not a direct inhibitor of the COOH-terminal cleaving enzyme, that nonglycosylated procollagen was converted by active enzymatic activity, and that medium from cells treated with tunicamycin did not contain the enzymatic activity. Therefore, a possible explanation for the inhibition of conversion by tunicamycin is that the COOH-terminal procollagen peptidease is a glycoprotein that is synthesized as an inactive zymogen and normally activated extracellularly. In the presence of tunicamycin, glycosylation of this protease is inhibited and this may result in impaired secretion of the enzyme. Alternatively, lack of glycosylation of the protease may directly affect its activity or may directly inhibit its activation.

The mechanism by which tunicamycin inhibits glycoprotein synthesis is not entirely understood; however, recent experiments with calf liver microsomes (16), chick embryo microsomes (17), and yeast protoplasts (18) have shown that tunicamycin specifically inhibits the formation of polysisoprenyl N-acetylgalactosaminyl pyrophosphate from UDP-N-acetylgalactosamine. Therefore, it is likely that tunicamycin will inhibit primarily the synthesis of those sugar side chains in glycoproteins which contain N-acetylgalactosamine. The inhibition of incorporation of mannose by tunicamycin strongly suggests that this saccharide follows N-acetylgalactosamine on the oligosaccharide side chain of procollagen. Indeed, in most glycoproteins in which carbohydrate is attached to asparagine, N-acetylgalactosamine linked N-glycosidically, is followed by mannos (40, 41). Clark and Kefalydis (9) have recently reported the presence of glucosamine, galactosamine, and mannos in type I procollagen isolated from chick tendon fibrils. Evidence against linkage of oligosaccharides to seryl or threonyl residues was provided, suggesting, by exclusion, linkage to asparaginyl side chains. The hypothesis that sugar moieties are necessary substituents of proteins designed for export from the cell (42) has been questioned (43). In this study, procollagen synthesized in the presence of tunicamycin was secreted normally by fibroblasts in culture and probably by cranial bone. Such procollagen can be assumed to lack oligosaccharides linked by asparagine-N-acetylgalactosamine bonds. Tunicamycin probably does not inhibit the addition of galactosyl and glucosyl moieties to hydroxylsyl residues in the triple helical region of the protein. The latter saccharides are probably not required for secretion of procollagen since secretion appears to be relatively normal in individuals who are deficient in lysyl hydroxide activity (44) and who therefore synthesize procollagen with very low levels of hydroxylsyl glycodies. Nevertheless, the requirement for some glycosylation of procollagen prior to secretion must be considered unsettled.

There is evidence that proteins destined for transmembrane transport are synthesized with short NH₂-terminal peptide extensions that facilitate the binding of ribosomes to the membrane of the endoplasmic reticulum and the subsequent transmembrane movement of the protein into the cisternal space (45). However, it is likely that other events modulate the progress of a protein in this secretory pathway. Preliminary
findings in this work suggest that the secretion of another major protein synthesized by fibroblasts is impaired by tunicamycin. This protein resembles the elastic fiber microfibrillar protein recently described by Muir et al. (35) and is thought to be related to cold insoluble globulin and fibroblast surface antigen. It is possible that some proteins, such as the elastic fiber microfibrillar protein, IgF (46), and perhaps the COOH-terminal procollagen propeptide are secreted through a glycosylation-dependent pathway whereas others can be secreted independently of glycosylation.

Many biologically active proteins contain oligosaccharide chain(s) (41). Some glycoproteins, like interferon (47), can undergo partial removal of the carbohydrate chain(s) by the action of glycosidases and remain active while others, such as human chorionic gonadotropin (48) and γ-globulin (49), lose normal activity when deglycosylated. The use of tunicamycin also raises the possibility that some proteins, such as the elastic fiber microfibril protein, IgG (46), and perhaps the COOH-terminal procollagen propeptide are secreted through a glycosylation-dependent pathway whereas others can be secreted independently of glycosylation.

Acknowledgments—The skillful technical assistance of Ms. Kathleen Williams-Geiger is gratefully acknowledged. We thank Dr. G. Tamura for his generous gift of tunicamycin. We also thank Drs. Peter Byers and Jeffrey Davidson for their helpful suggestions.

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* G. Balian and P. Bornstein, unpublished observations
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*J. Biol. Chem.* 1977, 252:955-962.

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