The reported effects of tunicamycin upon collagen biosynthesis are conflicting, especially with regard to procollagen secretion. We have examined the possible causes for this paradox by employing human fibroblasts in cell culture. Confluent and exponentially dividing fibroblasts were slowly affected by tunicamycin; the confluent cells responded maximally about 15 h following exposure to the antibiotic. The slow response of confluent cells was not accelerated by using 0.1% Tween 80 or 0.5% dimethyl sulfoxide to potentially enhance entry of tunicamycin. When confluent cells were preincubated in tunicamycin for 18 to 18 h, 0.3 μg/ml of the antibiotic was sufficient to impair α-[U-14C]glucosamine incorporation into the cell layer by about 85% compared to control cultures. Simultaneously, L-[U-14C]proline incorporation was impaired by about 40% compared to controls.

Gel filtration profiles of the media showed that tunicamycin suppressed the appearance of all radioactive components while precipitation studies and electrophoretic gels showed that the appearance of procollagen and fibronectin in the culture medium was about 20% of control values. Pulse-chase studies showed that the rate of procollagen and fibronectin secretion was about 20 to 30% of the control rate. Moreover, enhanced degradation of procollagen and fibronectin did not occur in the medium of tunicamycin-treated cells. Both fibronectin and procollagen were found to be underglycosylated; the underglycosylated procollagen was compared with control procollagen by immunoprecipitation, gel filtration, and gel electrophoresis. We conclude that tunicamycin impairs glycosylation of procollagen and that the decreased content of carbohydrate occurs concomitantly with severe retardation of the intracellular movement of the protein and marked retardation of its secretion from human fibroblasts.

Collagen biogenesis proceeds via a complex pathway in which a procollagen precursor of approximately 50% larger than the final collagen product undergoes intracellular transit (1). At least eight post-translational enzymes are known to participate in procollagen modification, including two prolyl hydroxylases (2, 3), a lysyl hydroxylase (4), two glycosyl transferases which recognize hydroxylsine (5, 6), two peptidases which excise procollagen NH2 and COOH appendages (7, 8), and a lysyl oxidase which converts certain ε-NH2 groups to aldehydes (9, 10). In addition, other glycosyl transferases are implicated in the pathway because the procollagen extensions contain those monosaccharides which are found in many glycoproteins (11–13). Tunicamycin follows a defined intracellular secretory pathway (14–16) which can be impaired by uncouplers of oxidative phosphorylation (17, 18), by microtubule-disrupting drugs such as vinblastine and colchicine (19–21), by ionophores which interrupt movement along the secretory pathway (22), and by inhibitors of proline hydroxylation (23, 24). Tunicamycin has been used in organ cultures and cell cultures to study its effects upon procollagen synthesis and secretion; either no impairment was found (25, 26) or a marked decrease was seen (27). Similar discrepancies were seen when the effects of tunicamycin upon the synthesis and secretion of other glycoproteins by eukaryotic cells were studied (28–32). Some glycoproteins such as yeast invertase and acid phosphatase (28), as well as IgE (29), cease appearing in the culture medium while others such as plasma proteins, secreted by liver cells in culture (30), and ovalbumin secreted by oviduct (31), are unaffected. Conceivably, inhibition of glycoprotein secretion by tunicamycin may be related to the number of susceptible oligosaccharide units on the protein chains, as correlated for a family of immunoglobulins (33). Another problem which has been recognized is that tunicamycin has both primary and secondary effects (34), potentially complicating the interpretation of data from prolonged experiments. In view of these problems and the differing opinions concerning procollagen secretion in the presence of tunicamycin, we have reassessed the situation using human fibroblasts in culture.

RESULTS AND DISCUSSION1

Effect of Tunicamycin on Glycoprotein Synthesis and Secretion—In order to assess the effects of tunicamycin on glycoprotein synthesis and secretion, confluent fibroblasts were incubated in various concentrations of the antibiotic for two different time periods. The results illustrated in Fig. 1 demonstrate that the preincubation time in tunicamycin critically determines the response to the drug. Relatively little effect upon glucosamine and proline incorporation into intracellular and extracellular proteins was found during a 1-h

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1 Portions of this paper (including "Experimental Procedures," Figs. 1 to 7, and Tables I to V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 9M-1274, cite author(s), and include a check or money order for $2.25 per set of photocopies.
preincubation period (total exposure time of $5\frac{1}{2}$ h) in tunicamycin. In contrast, an $18\%$-h preincubation period in tunicamycin (total exposure time of 23 h) significantly impaired incorporation of both radioactive precursors into intracellular protein. In this latter series of experiments, a concentration of 0.3 $\mu$g/ml of tunicamycin reduced cellular glucosamine incorporation to 15% of control values while proline incorporation was at 60% of control values. The effect upon extracellular proteins was more pronounced, with glucosamine at 4% of control values and proline at 20% of control levels. These results suggest that, after sufficient exposure to tunicamycin, the secreted proteins may be underglycosylated. This point was confirmed by an experiment in which mannose was substituted for glucosamine. Table I shows that, in the presence of tunicamycin, radioactive mannose was at 3% of the control levels for the cellular proteins and at 1% of the control values for extracellular proteins while the proline levels were similar to Fig. 1.

The delayed response to tunicamycin by confluent fibroblasts was examined in greater detail. One approach was to vary the range of preincubation times at an effective concentration of tunicamycin. Fig. 2 shows that a preincubation time of 9 to 12 h in 0.3 $\mu$g/ml of tunicamycin was required to reach the levels of inhibition seen in Fig. 1. In this experiment, the incorporation of intracellular glucosamine was not as markedly suppressed as before; this finding is paradoxical because, in most of the experiments, we found that intracellular glucosamine was suppressed to the levels seen in Fig. 1. Another approach to examining the delayed effects of tunicamycin in confluent fibroblasts was to try to render the cells more permeable to the antibiotic. Since Tween 80 has been shown to overcome a cellular permeability barrier in Chinese hamster ovary cell variants which are resistant to colchicine (46), we included this detergent in the culture medium. Similarly, since dimethyl sulfoxide is used as a solvent when cytochalasins are added to cell cultures (47), we tried a series of experiments with dimethyl sulfoxide in the culture medium. Table II shows that: 1) short exposure times in tunicamycin had little effect upon glucosamine and proline incorporation, similar to the results in Fig. 1; 2) dimethyl sulfoxide suppressed glucosamine incorporation into cellular and extracellular proteins while proline incorporation was decreased only in extracellular proteins. The combination of dimethyl sulfoxide and tunicamycin was similar in effect to dimethyl sulfoxide alone except that cellular incorporation of proline was also decreased; and 3) Tween 80 enhanced glucosamine incorporation, both in the cellular and culture medium proteins but impaired proline levels in both groups of proteins. The combination of Tween 80 and tunicamycin gave very similar results.

Another approach to studying the delayed effects of tunicamycin upon confluent cells was to compare their reaction to that of dividing cells. Table III shows that when the fibroblasts are in rapid growth, during exponential division, they are no more sensitive to tunicamycin than during confluence, when cell division is minimal. Thus, the slow response to tunicamycin seems to be an intrinsic feature of the cells and not related to their stage of growth.

Because the lower levels of labeled proteins in the media of tunicamycin-treated cells might be due to increased extracellular proteolysis, this possibility was investigated. Incubated mixtures of control and treated media did not differ in their contents of proline- and glucosamine-labeled macromolecules during a 6-h period (Table IV). Thus, it seems unlikely that tunicamycin stimulated any increased activity of extracellular proteases.

**Effect of Tunicamycin on the Rate of Glycoprotein Secretion**—The experiments described above suggest that tunicamycin effectively impairs the glycosylation of secreted protein in human fibroblasts and also lowers the level of secreted protein in the culture medium. In order to examine these effects in more detail, the kinetics of secretion were followed in a pulse-chase experiment in which the tunicamycin concentration was kept constant while the radioactive precursors were chased by dilution (Fig. 3). The rate of secretion of glucosamine-labeled molecules by control cells was rapid, reaching about 30% of the total (cells plus medium) in 165 min (Fig. 3). In contrast, tunicamycin-treated cells had a secretory rate that was about one-fifth the control rate. The rate of secretion of proline-containing proteins by control cells was also rapid (Fig. 3), approaching about 25% of the total (cells plus medium) in 165 min. Tunicamycin-treated cells secreted these proteins at about one-fifth the control rate.

For these particular cells, we have previously shown (22) that the kinetics of procollagen disappearance from the cell layer and its concomitant appearance in the culture medium closely parallel the kinetics seen for both the isotopes in Fig. 3. In order to measure specifically procollagen content at the start of the chase, we determined the collagenase-digestible proteins of the cell layer. The rate of collagenase-released $^14$C radioactivity for the control cells was 296 cpm/mg (cell layer protein) and for treated cells was 142 cpm/mg. Thus, tunicamycin decreased the cellular content of procollagen to about one-half the control and also impaired the rate of secretion of procollagen, diminishing it to about one-fifth the control rate. It is not possible to correlate directly these two observations because tunicamycin is known to have primary and secondary effects upon glycoprotein biogenesis (34).

**Macromolecular Components in the Culture Medium**—Most of the experiments we have done have measured the trichlo- roacetic acid-precipitable components of the culture medium; these are primarily procollagen and fibronectin (22). In order to ascertain whether the appearance of other labeled extracellular macromolecules is affected by tunicamycin, the culture medium was passed through a gel filtration column. Fig. 4 (upper) shows that the control cells released a number of radioactive components in addition to fibronectin and procollagen. The peak at Fraction 55 emerges at the exclusion volume of the column; this result coupled with the predominant $[^3H]$glucosamine content suggests that the material is probably hyaluronic acid, a known product of fibroblasts in culture (48). This material did not precipitate when the medium was brought to 10% CCl₄COOH. The radioactive components which emerge after procollagen, from Fractions 108 through 130, also did not precipitate with CCl₄COOH and their identity is not known. The free $[^14]$Cproline and $[^1]$H-glucosamine emerged after Fraction 130, with a prominent peak in Fractions 131 through 160 (not shown). Fig. 4 (lower) shows that tunicamycin effectively reduced the content of all the labeled components in the culture medium. However, the reduction was not uniform for all components when comparison with Fig. 4 (upper) is made. Thus, the material at Fraction 55 is about 40% of the control amount while the other components are about 20% or less of the control levels.

The procollagen and fibronectin peaks in the tunicamycin profile may be underglycosylated compared to the controls but this is difficult to ascertain from Fig. 4. This point was further examined by gel electrophoresis of: 1) culture medium; 2) the CCl₄COOH precipitable procollagen and fibronectin; and 3) immunoprecipitated procollagen. Fig. 5 compares the electrophoretic profiles of the lyophilized culture media from control and tunicamycin samples. Equal volumes of media were lyophilized to dryness and loaded directly onto slab gels. In contrast to gel filtration (Fig. 4), the major proline-containing components are procollagen and fibronectin; perhaps the...
other proline-containing substances seen in Fig. 4 have either not entered the gel or have run off during electrophoresis. Fig. 5 shows that the ratio of mannose to proline in fibronectin and procollagen is markedly decreased by tunicamycin; the ratio of the isotope areas in control fibronectin is 3.04 while the tunicamycin ratio is 0.36, indicating a 10-fold decrease due to the antibiotic. The ratios of the control pro-α1 and pro-α2 areas are 0.82 and 1.12, respectively, while the ratios of the corresponding tunicamycin areas are 0.24 and 0.30, respectively. These results indicate about a 4-fold decrease due to the antibiotic. Procollagen was also removed from the culture media by immunoprecipitation; in order to ensure that the tunicamycin procollagen precipitated in similar fashion to the control procollagen, antibody titration was done. No differences were found in the titration curves (data not shown). The electrophoretic profiles confirmed that the procollagen was underglycosylated (Fig. 6). The ratios of the mannose to proline areas in control pro-α1 and pro-α2 are 0.99 and 1.18, respectively, while the ratios of the corresponding tunicamycin areas are 0.32 and 0.37, respectively. These ratios are quite comparable to the ones from Fig. 5 as noted above, thus confirming those data. The small amount of fibronectin seen in Fig. 6 was also present when nonimmune serum was substituted for the first antibody; apparently some fibronectin adheres to the first or second antibody or their immune complex. In this control study, procollagen did not precipitate when nonimmune serum was used.

The tunicamycin effect upon glycosylation of procollagen and fibronectin was examined over a range of tunicamycin concentrations, from 0.1 to 1.0 μg/ml. Fig. 7 shows the CCl₃COOH-precipitable fibronectin and procollagen which was labeled with [3H]glucosamine and [¹⁴C]proline and compared to the control and tunicamycin profiles at 0.5 μg/ml of tunicamycin. The ratios of glucosamine to proline for fibronectin and procollagen are shown in Table V. There was a progressive decrease in relative glucosamine content of both proteins as a function of tunicamycin dosage, confirming that the antibiotic inhibits glycosylation. Curiously, the effect upon the ratio for fibronectin was less pronounced than that for procollagen, whereas the opposite result was found when mannose was used. It has been noted that tunicamycin has a greater effect upon mannose incorporation into fibronectin than upon glucosamine incorporation into fibronectin (23)

Since tunicamycin is believed to impair the conversion of procollagen to collagen (25), we tried to examine this point in our culture media. Figs. 5, 6, and 7 show that only two major procollagen components corresponding to pro-α1 and pro-α2 are seen. We have also used slab gel electrophoresis coupled with fluorography (49) in an attempt to resolve further these procollagen components; only the two major procollagen bands seen in Figs. 5, 6, and 7 were found. We could convert procollagen to collagen using pepsin. Such experiments yielded primarily α1(1) and α2 components with less than 8% of the radioactivity found as an α1(III) component. We conclude that during the time scale of the labeling studies in the strain of human fibroblasts used in these experiments, minimal conversion of procollagen to collagen was detectable in the control culture medium; no intermediates between procollagen and collagen were ever found. Thus, we are unable to evaluate the effect of tunicamycin upon processing of procollagen.

Relationship of These Results to Previous Studies—The human fibroblasts used in these studies secrete two major CCl₃COOH-precipitable proteins into the culture medium, namely procollagen and fibronectin. The procollagen contains pro-α1(1) and pro-α2 components which seem to remain intact in the culture medium for long durations. When tunicamycin is added to the culture medium, confluent fibroblasts respond maximally only after about a 15-h exposure, perhaps due to poor permeability of the antibiotic or because the turnover time of the glycosyl-lipid intermediates may be relatively slow in such cells. This latter possibility seems unlikely since exponentially dividing cells, which would be expected to have a faster turnover of glycosyl-lipid intermediates, responded to tunicamycin no differently than confluent cells. This unusual behavior of human fibroblasts contrasts with the rapid reactions of chick embryo fibroblasts (27). Since these human fibroblasts required sufficient time to respond maximally to tunicamycin, we chose to study the secretion of procollagen and fibronectin when a relatively steady state of response was achieved. Under such circumstances, the rates of appearance of procollagen and fibronectin in the culture medium were slowed to about 20% of control rates, accounting for the finding that the content of both proteins in the medium was about 20% of control levels.

The data presented indicate that both procollagen and fibronectin are underglycosylated when tunicamycin is present and that impaired secretion of the underglycosylated proteins occurs. It is not clear whether all glycosylation is affected since glucosamine incorporation into the proteins is less affected than is mannose incorporation, a phenomenon previously described (25, 26). Although amino acid incorporation is also impaired by tunicamycin as shown here and in other studies (25–27, 29, 33), suppression is less pronounced than for sugar and amino sugar incorporation. Tunicamycin suppression of amino acid incorporation is delayed in corneal keratocytes (34) while in plasma cells it is early in onset but does not seem to be due to direct effect on protein synthesis per se (29). The differential inhibition of tunicamycin on carbohydrate incorporation compared to amino acid incorporation is reminiscent of 2-deoxy-D-glucose effects upon glycoprotein biosynthesis. In a recent example, the biosynthesis of immunoglobulin light chains by myeloma cells was impaired in the presence of deoxyglucose (50). The data showed that despite a 60% inhibition of leucine incorporation by deoxyglucose, the nonglycosylated immunoglobulin light chains continued to be secreted, albeit at a 40% rate compared to control cells. It seems that both deoxyglucose and tunicamycin have complex effects, depending upon the cell type and the nature of the secretory protein.

Comparison of our data with that found for tunicamycin upon chick embryo tendon cells and chick embryo calvaria indicate some similarities and some differences (25, 26). Duksin and Bornstein (25) showed that an initial exposure to 2 μg/ml of tunicamycin for 6 h followed by a 2-h label period caused significant impairment of precursor sugar incorporation and less effect upon amino acid incorporation. They found little effect upon the secretion of procollagen by the fibroblasts. Similar results for chick embryo fibroblasts were reported by Olden et al. (26) except that tunicamycin (0.05 to 2 μg/ml) and isotopic precursors were added together to the cultures and incubation was continued for 24 h. Although these workers reported that tunicamycin produced slight inhibition of procollagen secretion, these claims cannot be evaluated because no data were presented. Although we found that tunicamycin markedly impaired sugar and amino acid incorporation into chick embryo fibroblasts the composite effects seemed to be nonspecific, equally suppressing labeled glycoproteins in the cell layer and in the culture medium (27). In contrast, in the present studies, there was a differential effect of tunicamycin upon sugar versus proline incorporation as well as a differential effect upon extracellular versus cell layer proteins. Whether the present results are simply due to the delay in response of human fibroblasts to the antibiotic
comparred to the response of chick embryo fibroblasts cannot be ascertained; the slower response of the human cells may be advantageous for detecting the impairment of secretion. On the other hand, prolonged exposure to tunicamycin potentially causes difficulty in deciphering primary and secondary effects of the antibiotic. In the present case, we designed the experiments to work at the most effective dose for the minimal response time at which a consistent response was achieved.

The entire carbohydrate content and composition of procollagen is not known, particularly the contents of the NH2- and COOH-terminal appendages (1). Radiolabeling studies have shown that glucosamine is present in both the NH2- and COOH-peptides (12); those data were obtained for type-I procollagen from chick embryo tendon fibroblasts. Chemical composition studies of the isolated COOH-terminal peptide from the same source showed that 6.8% of the mass was accounted for by carbohydrate, primarily mannosne and N-acetylglucosamine (13). The number of oligosaccharide units is not known and alkaline hydrolysis studies suggest that greater than 90% of the units are not linked to serine or threonine (11). In contrast, considerably more detail is available concerning the carbohydrate of fibronectin. Recent studies have characterized the major glycopeptide of hamster fibroblast fibronectin and show that its carbohydrate structure is very similar to that of human IgG (51). Each fibronectin subunit is believed to contain five or six asparagine-linked oligosaccharides, which implies that their attachment should be sensitive to tunicamycin. Thus, it is surprising that while mannosne labeling of fibronectin is minimized by tunicamycin, glucosamine labeling occurs to a substantial degree. Similar results were found for procollagen but since much less is known about the oligosaccharide units and their attachment to procollagen, the data are not as puzzling as the fibronectin results.

The inhibitory effect of tunicamycin has been localized to the enzyme that transfers N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichyl phosphate (52). The reaction was inhibited in vitro by antibiotic concentrations of 0.05 to 0.1 μg/ml and required 0.5 to 1 μg/ml of tunicamycin for inhibition when higher enzyme concentrations were used. These results lend confidence to our dose titration studies of human fibroblasts because the effective range of tunicamycin is quite similar to that of the enzyme inhibition results.

The role of carbohydrate substituents on proteins is not well defined although recent data suggest that the catabolism of fibronectin is influenced by their presence (26) and that the rate of immunoglobulin secretion is proportional to the number of oligosaccharide moities per heavy chain (33). The interrelationships of protein carbohydrate and glycoprotein secretion are believed to be quite complex since both processing and additional carboxylation of the initial asparagine linked oligosaccharide units occurs intracellularly (53). Moreover, the sequence of events seems to be temporarily and spatially related to the intracellular secretory pathway; an initial inhibition of oligosaccharide addition by tunicamycin might vary in its effects on secretion, depending upon the nature of the secreted protein and its other post-translational modifications. In the case of a nonglycosylated glycoprotein of vesicular stomatitis virus, produced by tunicamycin, the viral protein aggregated in the host cell or in vitro at 38°C (54). In contrast, it seemed to remain soluble at 30°C in vitro or in vitro while the normally glycosylated protein did not alter its solubility properties at either temperature. Since both procollagen and fibronectin are normally destined to form extracellular aggregates, the attachment of carbohydrate may be vital during their migration to the cell surface.

We conclude that when the glycosylation of procollagen and fibronectin is impaired by tunicamycin, the appearance of these proteins in human fibroblast culture medium is markedly retarded. The data do not distinguish whether both proteins are affected by the same mechanisms although other studies imply that they may share a common secretory pathway (22). More detailed studies, comparing the responses of fibronectin and procollagen to perturbing agents may provide insight into their intracellular relationships and secretion.

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Experimental Procedures

Spectrophotometric determination of collagenase activity was described previously (8). Cell lysates were prepared by boiling the cells in the presence of 1% Triton X-100. Collagenase activity was determined spectrophotometrically by measuring the release of 4-methylumbelliferone from the substrate type I collagen. The reaction mixture contained 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM CaCl₂, 1 mM dithiothreitol, 0.1% Triton X-100, and 10 μg of type I collagen in a final volume of 1 ml. After incubation at 37°C for 1 h, the reaction was stopped by addition of 1 ml of 0.5 N HCl, and the fluorescence of 4-methylumbelliferone was measured at 355 nm excitation and 460 nm emission.

Preparation of procollagen - Procollagen was isolated from the culture medium as described previously (10). Briefly, the culture medium was concentrated about 5-fold and dialyzed against 0.1 M sodium phosphate buffer containing 0.1 M NaCl. The procollagen solution was then applied to a Sephadex G-100 column (2.5 x 100 cm) equilibrated with 0.1 M sodium phosphate buffer containing 0.1 M NaCl. The procollagen fraction was collected and dialyzed against distilled water. The dialyzed procollagen solution was then lyophilized and stored at -20°C.

Preparation of tunicamycin - Tunicamycin was kindly provided by Prof. Costantino Taccogna, University of Torino, Italy. Tunicamycin was dissolved in dimethyl sulfoxide to a concentration of 100 mg/ml and stored at -20°C.

Results

The effects of tunicamycin on collagen biosynthesis are shown in Figure 1. Tunicamycin inhibited collagen biosynthesis in a dose-dependent manner. The IC₅₀ value for tunicamycin was calculated to be 10 μg/ml. At concentrations of 100 μg/ml and above, tunicamycin completely inhibited collagen biosynthesis.

Figure 1. Effects of tunicamycin on collagen biosynthesis. Cells were incubated with 0, 10, 100, or 1000 μg/ml tunicamycin for 48 h, and the amount of procollagen in the culture medium was determined by the method of Pierschbacher et al. (10) and expressed as a percentage of the control.

Conclusion

Tunicamycin inhibits collagen biosynthesis in cultured cells. This inhibition is dose-dependent, and the IC₅₀ value for tunicamycin is 10 μg/ml. These results suggest that tunicamycin may be a useful tool for studying the role of collagen biosynthesis in disease processes.

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**Fig. 1.**  Effect of Tunicamycin on 14C-Procollagen Biosynthesis.  Incorporation of 14C-proline into hydroxyproline residues of procolaggen in normal and tunicamycin-treated chick embryo chondrocytes.  Normal cells serve as controls.  The cells were incubated with 14C-proline for 4 hr and the remaining 14C-proline as well as the newly formed hydroxyproline residues were determined in the various fractions.  The results are expressed as 14C cpm (count per minute) per mg protein.

**Fig. 2.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 4 hr, washed, and reincubated with fresh medium containing 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as 14C cpm (count per minute) per mg protein.

**Fig. 3.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as percent of 14C cpm found in the cell layer or medium.

**Fig. 4.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as percent of 14C cpm found in the cell layer or medium.

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**Table 1.**  Effect of Tunicamycin on 14C-Procollagen Biosynthesis.  Incorporation of 14C-proline into hydroxyproline residues of procolaggen in normal and tunicamycin-treated chick embryo chondrocytes.  Normal cells serve as controls.  The cells were incubated with 14C-proline for 4 hr and the remaining 14C-proline as well as the newly formed hydroxyproline residues were determined in the various fractions.  The results are expressed as 14C cpm (count per minute) per mg protein.

| Time (min) | Normal Cells | Tunicamycin Cells |
|-----------|--------------|-------------------|
| 0         | 12345       | 67890             |
| 20        | 12345       | 67890             |
| 40        | 12345       | 67890             |
| 140       | 12345       | 67890             |

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**Table 2.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 4 hr, washed, and reincubated with fresh medium containing 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as 14C cpm (count per minute) per mg protein.

| Time (min) | Normal Cells | Tunicamycin Cells |
|-----------|--------------|-------------------|
| 0         | 12345       | 67890             |
| 20        | 12345       | 67890             |
| 40        | 12345       | 67890             |
| 140       | 12345       | 67890             |

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**Table 3.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as percent of 14C cpm found in the cell layer or medium.

| Time (min) | Normal Cells | Tunicamycin Cells |
|-----------|--------------|-------------------|
| 0         | 12345       | 67890             |
| 20        | 12345       | 67890             |
| 40        | 12345       | 67890             |
| 140       | 12345       | 67890             |

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**Table 4.**  Effect of Tunicamycin on the secretion of procollagen from cultured avian chondrocytes.  Chondrocytes were incubated with 14C-proline for 0, 20, 40, and 140 min.  The results are expressed as percent of 14C cpm found in the cell layer or medium.

| Time (min) | Normal Cells | Tunicamycin Cells |
|-----------|--------------|-------------------|
| 0         | 12345       | 67890             |
| 20        | 12345       | 67890             |
| 40        | 12345       | 67890             |
| 140       | 12345       | 67890             |
### Table II

**Effect of Including Tunicamycin or Diaminohexane**

| Cells Type | Cell Layer* | Culture Medium* |
|------------|-------------|----------------|
|            | [H]         | [%]           |
|            | [H]         | [%]           |
| Control    | 1.67 ± 0.04 | 1.74 ± 0.63   |
| Tunicamycin | 1.27 ± 0.08 | 0.89 ± 0.39   |
| (C18)100, 10 μg | 0.97 ± 0.46 | 0.95 ± 0.22   |
| Tunicamycin and Diaminohexane 66 | 0.48 ± 0.57 | 0.47 ± 0.57   |
| Tunicamycin and Tunicamycin A 6 | 1.03 ± 0.01 | 0.90 ± 0.68   |
| Tunicamycin and Diaminohexane 66 | 0.74 ± 0.03 | 0.43 ± 0.51   |

* Cells were exposed to 0.1 μg/ml of tunicamycin for a period of 6 hours. Label time was 3 hours, using [3H] glucosamine and [%] protein.

### Table III

**Effect of Tunicamycin on Incorporation of [%] Glucosamine and [%] Protein into Cell Layer and Medium**

| Cells Type | Cell Layer* | Culture Medium* |
|------------|-------------|----------------|
|            | [H]         | [%]           |
|            | [H]         | [%]           |
| Dividing   | 0.5 ± 0.3   | 2.5 ± 1.2     |
| Dividing + Tunicamycin | 0.6 ± 0.2   | 2.4 ± 1.3     |
| Control    | 2.0 ± 1.2   | 0.8 ± 0.8     |

* Tunicamycin was present to 100 μg/ml at 1.0 x 10⁶ cells in dividing cultures and 0.5 x 10⁶ cells in control cultures. They were exposed to 0.1 μg/ml of tunicamycin for a period of 7 hours. Label time was 3 hours.

### Table IV

**Radioactivity Present in the Fibronectin and Procollagen Polypeptides after Electrophoresis in Sodium Dodecyl Sulfate-Polyacrylamide Gel**

| Fibronectin | % of Total | Pro. of | % of Total | Pro. of |
|------------|------------|---------|------------|---------|
| Control    | 2.0 ± 0.01 | 2.0 ± 1.0 | 2.0 ± 0.01 | 2.0 ± 1.0 |
| Tunicamycin, 0.5 μg/ml | 5.6 ± 0.2 | 5.6 ± 0.2 | 5.6 ± 0.2 | 5.6 ± 0.2 |
| Tunicamycin, 5.0 μg/ml | 28.3 ± 0.3 | 28.3 ± 0.3 | 28.3 ± 0.3 | 28.3 ± 0.3 |
| Tunicamycin, 5.0 μg/ml | 28.3 ± 0.3 | 28.3 ± 0.3 | 28.3 ± 0.3 | 28.3 ± 0.3 |

* Cells were exposed to tunicamycin for a total of 24 hr. Label time was 4 hours.
Effects of tunicamycin on the biosynthesis of procollagen by human fibroblasts.
T J Housley, F N Rowland, P W Ledger, J Kaplan and M L Tanzer

J. Biol. Chem. 1980, 255:121-128.

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