EFFECTS OF ANTIMICROTUBULAR AGENTS ON THE SECRETION OF COLLAGEN

A Biochemical and Morphological Study

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ABSTRACT

Embryonic chick cranial bone was cultured in the presence of the antimicrotubular agents, colchicine and vinblastine, and with a number of other compounds known from previous studies to affect the cellular handling of collagen. Secretion of procollagen, quantitated by light microscope autoradiography, was correlated with the extent of conversion of procollagen to collagen and with rates of collagen and noncollagen-protein synthesis.

Colchicine inhibited procollagen secretion and conversion to collagen and specifically inhibited collagen synthesis. Cells exposed to colchicine revealed an increased number of dilated Golgi-associated vacuoles and vesicles, some of which contained parallel aggregates of filamentous structures. These observations suggest that the pathway of at least a fraction of procollagen secretion by osteoblasts includes the Golgi complex. Disruption of microtubules may interfere with the movement of Golgi-derived vesicles, and the resulting accumulation of collagen precursors in the Golgi complex may lead secondarily to an inhibition of synthesis. Although vinblastine also inhibited both procollagen secretion and conversion to collagen, the observed reduction in general protein synthesis and striking changes in the ultrastructure of the rough endoplasmic reticulum complicated interpretation of the effects.

Interpretation of the effects of cytochalasin B was limited by the finding that the cellular response in cranial bone was markedly heterogeneous and that, contrary to some previous reports, the drug caused an inhibition in the incorporation of radiolabeled amino acids into both collagen and noncollagen protein.

INTRODUCTION

Collagen exists within cells as a higher molecular weight precursor, procollagen, which contains additional sequences at the NH_2-termini of all three chains of the macromolecule (see references 3 and 42 for a review). The subcellular mechanisms involved in the transport of procollagen from its

site of synthesis on the rough endoplasmic reticulum (RER)[1] to the extracellular space are not well understood (4, 38). Thus, in contrast to the process in exocrine glands (7, 22), the role of the Golgi complex in the secretion of collagen and other connective tissue proteins has not been firmly established. There is evidence that secretion of a fraction of collagen by fibroblasts may be achieved by bypassing the Golgi complex, possibly by intermittent communication via vesicles or direct communication of the cisternal space of the RER with the exterior of the cell (40). However, recent electron microscope studies of the corneal epithelium (20, 44) and odontoblasts, osteoblasts and fibroblasts (48, 49) have identified smooth-surfaced Golgi-associated vesicles and vacuoles containing material which may be collagen.

If the transcellular movement of procollagen involves packaging within membrane-bound structures, such movement may be mediated by microtubular elements. Communication between cisternal elements or vesicles and the extracellular space may be dependent on the ability of microtubules to assist in the translocation of these structures to positions subjacent to the plasma membrane. Colchicine and vinblastine were found to inhibit secretion of procollagen by fibroblasts (9, 10), and these compounds, as well as other antimicrotubular agents, retarded the conversion of procollagen to collagen by cranial bones in culture (12). Since an extracellular site for procollagen conversion is likely (3, 27, 42), these studies implicate microtubules in the transcellular movement of procollagen. However, the effects of antimicrotubular agents on secretory processes are complex, and metabolic actions unrelated to microtubular disruption have been identified (see Discussion).

The present study combines a biochemical, autoradiographic, and electron microscope study of the effects of antimicrotubular agents upon cranial bone cells in organ culture. Secretion, assessed by light microscope autoradiography, was correlated with the rate of collagen and noncollagen-protein synthesis as determined by incorporation of radiolabeled amino acids into these protein fractions. The effect of cytochalasin B, a compound previously found to inhibit collagen but not noncollagen-protein synthesis (12), has also been examined in greater detail.

[1] *Abbreviations used in this paper:* RER, rough endoplasmic reticulum: DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; m-Cl-CCP, carbonyl cyanide m-chlorophenylhydrazone.

MATERIALS AND METHODS

Tissue Culture of Cranial Bone

17-day old chick embryos were obtained from a local hatchery. Cranial (frontal and parietal) bones were dissected out as a single sliver of bone and incubated (3 calvaria/6 ml) for 40 min at 37°C in Dulbecco's modification of Eagle's medium further modified as previously described (5). The bones were then transferred to fresh medium containing the test agent and incubated for 20 min. At this time L-[2,3-^{3}H]proline (50 Ci/mmol, New England Nuclear, Boston, Mass.) was added at a concentration of 16 μCi/ml and incubation continued for an additional 20 min. For pulse-chase experiments, calvaria were washed once with fresh medium and incubated for a third 20 min period in the presence of the test agent and 1.8 mg/ml of L-[^{1}H]proline. Vinblastine (2×10^{-5} M), colchicine (1×10^{-4} M), cis-hydroxyproline (400 μg/ml), α,α'dipyridyl (1.4×10^{-3} M), dibutyryl cyclic AMP (1×10^{-3} M), and m-Cl-CCP (0.2 μg/ml) were added directly to the incubation medium. In experiments with cytochalasin B the compound was dissolved at a concentration of 1 mg/ml in 10% dimethyl sulfoxide (DMSO) and used in the incubation medium at a concentration of 10 μg/ml in 0.1% DMSO. Control cultures contained the same concentration of DMSO.

In order to test the effects of antimicrotubular agents or cytochalasin B on collagen and noncollagen-protein synthesis, cranial bones were preincubated as described above and then incubated for 20 min in the presence of 1×10^{-4} M colchicine, 5 μg/ml of cytochalasin B, or concentrations of vinblastine ranging from 2×10^{-6} M to 2×10^{-5} M. Cytochalasin B was dissolved at a concentration of 1 mg/ml in 70% ethanol, yielding a final concentration of ethanol in the tissue culture medium of 0.35%. Control cultures contained the same concentration of ethanol. [^{3}H]proline or [^{3}H]tryptophan (5 Ci/mmol) was then added at concentrations of 8 μCi/ml or 3.2 μCi/ml, respectively, and the incubation continued for an additional 15–90 min.

Extraction Procedures

The incubations were terminated by washing bones on a Buchner funnel with three portions of ice-cold 0.15 M NaCl.

PROCOLLAGEN: A section of bone adjacent to the suture line separating the frontal and parietal bones was removed for autoradiography and electron microscopy. The bones were then homogenized in 10 ml of 0.5 M acetic acid containing 0.1% Triton X-100 and extracted with this solution at 4°C for 24 h. Extracts were clarified by centrifugation at 39,000 *g* and dialyzed against 0.08 M sodium acetate, pH 4.8, at 4°C.

TOTAL PROTEIN: Bones were homogenized in 10 ml of cold H_2O and the extracted protein was precipitated by addition of trichloroacetic acid (TCA) to a concentration of 10% and tannic acid to 0.25%. Other protein precipitants such as 90% ethanol and 10% TCA alone were tested but proved less satisfactory. The homogenate together with precipitated protein was centrifuged at 2,500 *g* for 10 min, washed with 10% TCA, 0.25% tannic acid, and recentrifuged. These procedures were repeated two additional times. The pellet was then suspended in 5 ml of 0.1 M NaOH and heated to 55°C for 10 min to destroy aminoacyl tRNA. The suspension was neutralized with 4 M HCl and the protein reprecipitated with 10% TCA, 0.25% tannic acid.

Amino Acid Analyses

[^{3}H]Proline-labeled material was hydrolyzed in 6 ml of 6 N HCl for 24 h at 108°C. Hydrolysates were flash evaporated, dissolved in 5 ml of H_2O, and centrifuged at 2,500 *g*. Proline and hydroxyproline were quantitated in 17 different samples of chick cranial bone using an amino acid analyzer. The molar ratio of hydroxyproline to proline was found to be 0.584 ± 0.035. In subsequent analyses, hydroxyproline was quantitated by a modification of the automated colorimetric method of Grant (18) in which Brij 35 was added to the chloramine T solution. The proline content in these samples was then calculated by using the formula:

$$\text{proline } (\mu\text{mol}) = \frac{\text{hydroxyproline } (\mu\text{mol})}{0.584}$$

Radioactive proline and hydroxyproline were separated using a column (0.9 × 17 cm) of Aminex Q-150S (Bio-Rad Laboratories, Richmond, Calif.) eluted with 0.2 N sodium citrate (pH 2.98), or a column (0.9 × 15 cm) of AG 50W-X8 resin, 200–400 mesh (Bio-Rad) eluted with 0.2 N Na citrate, pH 3.25. Total radioactivity in proline and hydroxyproline was determined by counting carefully measured aliquots of column effluents in 10% Bio-Solv-3 (Beckman) (5). Recovery of the radioactivity applied to columns in peaks coeluting with proline and hydroxyproline exceeded 90% in all cases.

If it is assumed that the contents of proline and hydroxyproline in collagen are approximately equal and that the extent of hydroxylation was not affected by the chemical agents used in these experiments (10, 12), then the specific activity of proline in noncollagenous protein can be obtained by subtracting the values determined for counts per minute and micromoles of hydroxyproline from the corresponding values determined for proline.

[^{3}H]Tryptophan-labeled material was hydrolyzed by NaOH in the presence of partially hydrolyzed starch (21). After hydrolysis the samples were neutralized with HCl and made up to 10 ml total volume with 0.2 N sodium citrate buffer, pH 4.25. One such sample was analyzed for tryptophan by elution from a column (0.9 × 12 cm) of PA 35 resin (Beckman Instruments, Inc., Fullerton, Calif.) eluted with 0.2 N sodium citrate, pH 5.4, in order to verify that radioactivity was limited to the position of elution of authentic tryptophan. The total protein in hydrolysates containing [^{3}H]tryptophan was determined by fluorometric analysis of amino acids using the fluorescamine reagent (46). Since tryptophan constitutes only a minor component of the additional sequences in procollagen (47), the activity of tryptophan (expressed as counts per minute/micromole of total amino acids) provides another measure of noncollagen-protein synthesis.

Conversion of procollagen to collagen

Extracts of cranial bones containing procollagen and collagen were chromatographed on CM-cellulose at 40°C in 0.04 M sodium acetate, pH 4.8, containing 4 M urea as previously described (1). Under these conditions the proα1 and α1 chains are well separated, and the ratio of counts in the two chromatographic fractions provides a measure of the relative contents of the precursor and product since proα1 and α1 are characteristic of acid-extracted procollagen and collagen, respectively. Recently, several laboratories have demonstrated that procollagen is synthesized with disulfide bonds linking the three chains and that acid extraction fosters limited proteolysis by tissue proteases, leading to a loss of disulfide-bonded regions (3, 6, 16, 31, 42, 43). However, during prolonged extraction and subsequent preparation of acid-treated bone procollagen, all procollagen fractions containing interchain disulfide bonds are converted to a form of the precursor in which chains derived from proα1 elute reproducibly from CM-cellulose in a position preceding α1 (1, 31). Division of the ratio proα1/α1 in chromatograms obtained under experimental conditions by the same ratio from control chromatograms, therefore, provides an indication of the extent to which a test substance affects the conversion of procollagen.

Autoradiography and Electron Microscopy

The tissues were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 6 h at 4°C. They were then washed briefly in buffer, postfixed in 2% osmium tetroxide, buffered with *S*-collidine (pH 7.3) for 1 h, dehydrated through a graded series of ethanol, and subsequently infiltrated and embedded in epoxy resin (40).

Cross sections of the bones were made at approximately 1-μm thickness and were stained with azure II-methylene blue for routine examination. A series of unstained sections from each set of bones from each experiment were mounted on glass slides and subsequently coated with a dipping machine (25) with Eastman Kodak NTB-11 emulsion (Eastman Kodak Co., Rochester, N.Y.). Slides were developed in Dektol after being exposed for 12 wk, fixed in Kodak acid fixer, and then stained with azure II-methylene blue.

Random light micrographs were taken with an oil emersion lens of the regions demonstrating osteoid formation. These were then counted by the method previously used by Ross and Benditt (40) in terms of intracellular vs. extracellular localization of silver grains.

Sections for electron microscopy were cut at approximately 100 nm, stained with lead tartrate followed by uranyl acetate, and examined with an AEI-EM 801 electron microscope.

RESULTS

Correlation between Secretion and Conversion of Procollagen to Collagen

Colchicine and vinblastine inhibited the conversion of procollagen to collagen and produced a parallel reduction in the extent to which procollagen is secreted, as judged by the proportion of silver grains present over cells compared with the extracellular matrix in autoradiograms of proline-labeled material (Table I). As positive controls the following compounds were used: cis-hydroxyproline which is incorporated into collagen as an analogue of proline and inhibits secretion of the protein (37), α,α'dipyridyl which inhibits peptidyl hydroxylation and collagen secretion (23), and m-Cl-CCP, an inhibitor of oxidative phosphorylation. These compounds both increased the proportion of proline-labeled material retained intracellularly and inhibited procollagen conversion (Table I). Since recent observations indicate that the conversion of procollagen to collagen occurs extra-

TABLE I

*Conversion of Procollagen to Collagen and Secretion of Procollagen Determined by the Ratio of Proα1 to α1 Chain and the Proportion of Silver Grains within the Confines of Cells, Respectively**

	Proα1 / α1	Exp/control	Total grains counted	Grains intracellular (%)
Exp 1				
Control	0.40	1.0	10,129	40
vinblastine 2×10^{-5} M	1.66	4.15	2,984	71.3
cis-hydroxyproline 400 μg/ml	0.74	1.85	2,955	65.7
α,α'dipyridyl 1.4×10^{-3} M	1.12	2.80	2,854	73.9
Dibutyryl cyclic AMP 1×10^{-3} M	0.31	0.78	9,748	35.8
Exp 2				
Control	0.36	1.0	—	—
m-Cl-CCP‡ 0.2 μg/ml	1.24	3.45	4,649	64.4
Colchicine 1×10^{-4} M	1.79	4.97	1,543	76.6
Exp 3				
Control (0.1% DMSO)	0.54	1.0	7,519	46.6
Cytochalasin B 10 μg/ml in 0.1% DMSO	0.62	1.15	11,998	57.3
Cytochalasin B§ 10 μg/ml in 0.1% DMSO	0.62	1.15	10,304	58.4

* Cranial bones were preincubated for 40 min. The bones were then transferred to medium with the test substance. After 20 min, a proline label was introduced for 20 min. Thereafter the label was removed and the tissue chased with an excess of unlabeled proline for 20 min in the continued presence of the drug. See text for details.

‡ Drug present only during chase.

§ No drug present during 20-min chase.

cellularly (3, 27, 42), inhibition of secretion would result in an increase in the ratio of proα1/α1 chain, as measured chromatographically.

Dibutyryl cyclic AMP produced only a small change (an acceleration) in the conversion of procollagen to collagen, and the proportion of grains found intracellularly was also close to that found in control sections, although again the deviation was toward an augmentation in secretion. Results of a similar nature were reported by Dehm and Prockop (9) working with freshly isolated tendon fibroblasts. The significance of these changes is difficult to assess and additional experiments which take into account tissue levels of cyclic AMP as well as the turnover of the nucleotide will be required.

Cytochalasin B had previously been found in pulse label experiments to enhance conversion of procollagen to collagen (12). No such enhancement was evident in experiments in which a pulse-chase protocol was followed (Table I). Nevertheless, a moderate degree of intracellular retention of procollagen is indicated by the grain count.

Effects on Collagen and Noncollagen-Protein Synthesis

In order to further evaluate these findings, the effects of colchicine, vinblastine, and cytochalasin B on collagen and noncollagen synthesis by cranial bone were examined. Vinblastine, in concentrations of 2×10^{-6} or 2×10^{-5} M, reduced both noncollagen-protein and collagen synthesis by approximately 50%, as indicated by incorporation of labeled proline (Table II). These results, obtained during the course of a pulse period of 30 min after a 20-min preincubation in the test agent, were confirmed by experiments in which the pulse period varied from 20 to 60 min (Fig. 1). Similarly, cytochalasin B (5 μg/ml) inhibited both noncollagen and collagen synthesis by about 25–30% (Table II, Fig. 1). The effects of these compounds on general protein synthesis, indicated by a reduction in proline incorporation, were confirmed by a similar reduction in tryptophan incorporation (Table III).

In any study in which a labeled precursor is used to monitor the cellular synthesis of a macromolecule, the consequences of changes in the transport of the precursor across the cell membrane must be considered. It is now well established that cytochalasin B inhibits the cellular uptake of nucleosides and sugars (13, 24, 29, 33).

TABLE II

*Protein Synthesis by Cranial Bone in the Presence of Vinblastine and Cytochalasin**

	Total proline incorporation‡ percent of control	Noncollagen protein proline‡ percent of control	Hydroxyproline‡ percent of control
Exp 1			
Control	100	100	100
Vinblastine			
2×10^{-6} M	51	59	57
2×10^{-5} M	55	64	58
2×10^{-5} M	47	51	54
2×10^{-5} M	50	43	49
Exp 2§			
Control	100	100	100
Cytochalasin B			
5.0 μg/ml	80	80	87
5.0 μg/ml	73	75	81
5.0 μg/ml	75	70	75

* Tissues were incubated for a total of 50 min in the test agent. Proline label was added during the last 30 min of this period.

‡ Expressed as counts per minute per micromole of amino acid.

§ Control and experimental flasks contained 0.35% ethanol.

Such effects may be responsible for the reported inhibition by cytochalasin B of mucopolysaccharide synthesis (41). No specific information is available, however, on the effect of this compound on amino acid transport. We attempted to evaluate the effect of cytochalasin B on proline transport in cranial bone, but the limitations imposed by difficulty in separation of cellular and extracellular spaces in this tissue have not permitted a clearcut interpretation of the results.

In contrast to vinblastine and cytochalasin B, colchicine, at a concentration of 1×10^{-4} M, had no significant effect on proline incorporation into noncollagen-protein (Fig. 2) and none on general protein synthesis as indicated by tryptophan incorporation (Table III). However, at the conclusion of a 90-min pulse (and a total of 110 min in the presence of the compound), collagen synthesis was reduced by more than 30% as indicated by the reduction in the specific activity of hydroxyproline (Fig. 2).

Fine Structure

The ultrastructural alterations observed in vinblastine-treated calvaria involved most of the

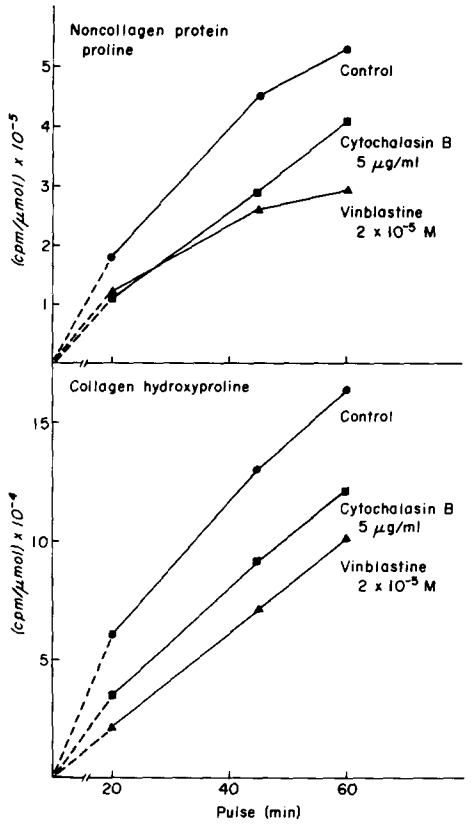

FIGURE 1 Incorporation of proline into noncollagen protein and collagen hydroxyproline by cranial bone in the presence of vinblastine or cytochalasin B. Bones were preincubated with or without the test agent for 20 min and then pulsed with 16 μCi/ml of L-[^{3}H]proline for 20–60 min. Data are expressed as the specific activities of proline and hydroxyproline.

cytoplasmic constituents. Aggregates of membranous structures, presumed to be analogous to the crystalloid structures previously reported (2), were present in several of the cells (Figs. 3 and 4). Typical vinblastine-induced crystalloid aggregates were also seen. Equally striking were the alterations seen in the RER. The cisternae of the RER were vesicular in appearance (Figs. 3 and 4), in contrast to the long, interconnecting channels usually characteristic of osteoblasts and fibroblasts (39). The Golgi complex of these cells was small, contained few large vacuoles, and had larger than usual numbers of small associated vesicles. No microtubules were seen in any of the cells examined.

Osteoblasts and fibroblasts in colchicine-treated bones, in contrast to those exposed to vinblastine, showed striking alterations in the Golgi complex.

TABLE III

Protein Synthesis as Determined by Incorporation of Tryptophan

	Percent of control*
Exp 1	
Vinblastine 2×10^{-5} M	66
Cytochalasin B 5 μg/ml	79
Exp 2	
Vinblastine 2×10^{-5} M	56
Cytochalasin B 5 μg/ml	62
Colchicine 1×10^{-4} M	118

* The data were determined as counts per minute/micromole of total amino acids.

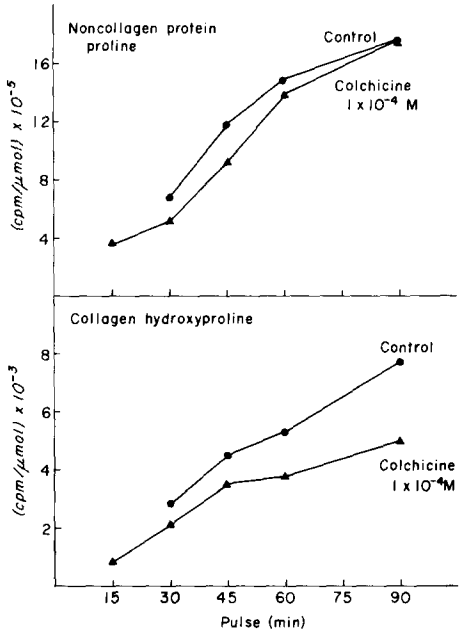

FIGURE 2 Incorporation of proline into noncollagen protein and collagen hydroxyproline by cranial bone in the presence of colchicine. See legend to Fig. 1 for additional details.

The rough endoplasmic reticulum of these cells was usual in appearance and the normal pattern of attached ribosomes, consisting of long parallel and curved arrays, was also present. The principal change observed in the Golgi complex of the colchicine-treated cells consisted of an increased number of Golgi-associated vesicles and vacuoles.

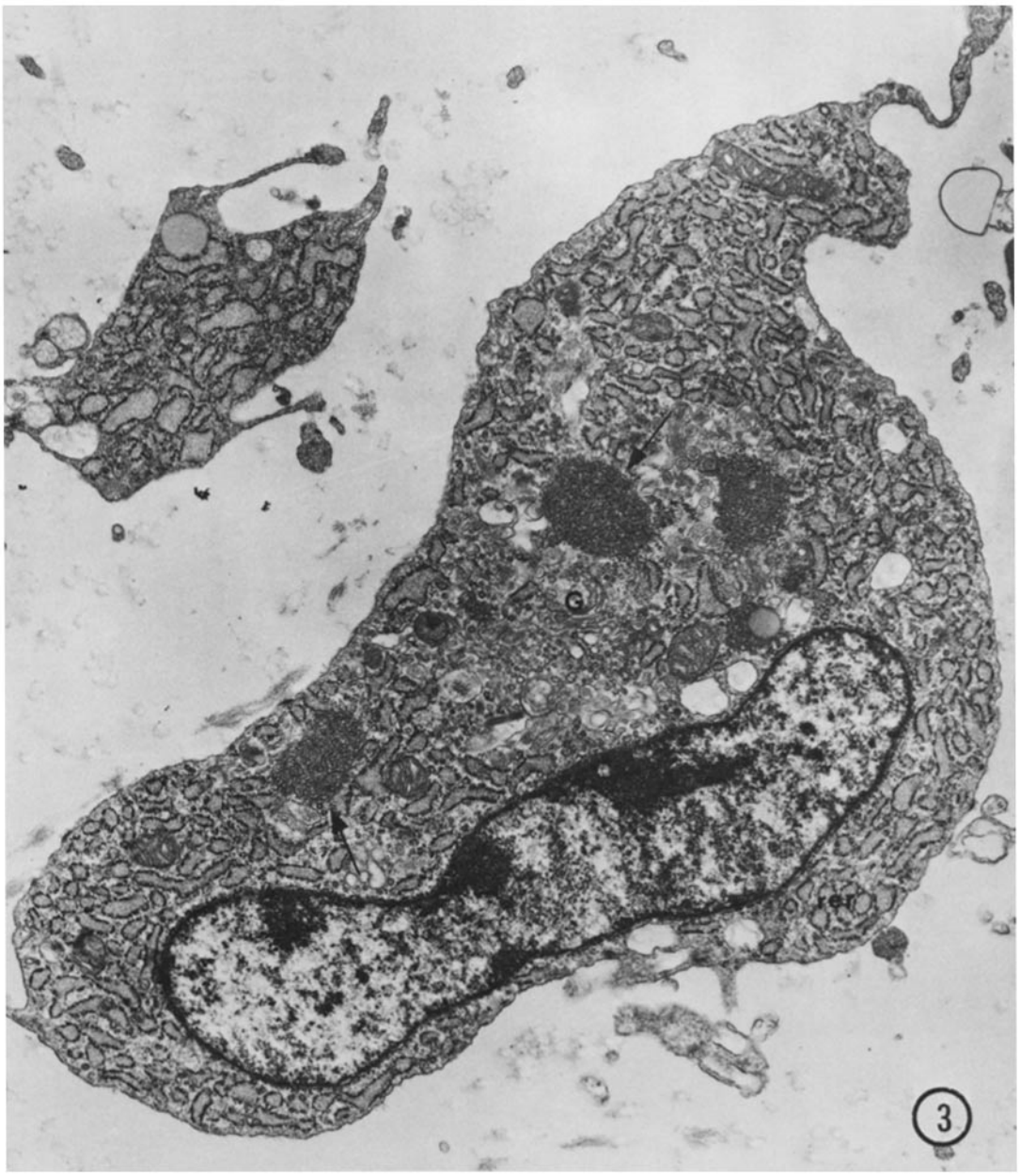

FIGURE 3 This electron micrograph represents a periosteal fibroblast after treatment with vinblastine. Several membranous aggregates (arrows) can be seen. In addition, the cisternae of the rough endoplasmic reticulum appear to be vacuolar and to have lost their usual three-dimensional canalicular appearance (*rer*). The Golgi complex (*G*) appears unaltered in this micrograph. × 14,000.

Many of these were quite large (up to 0.7 μm) (Fig. 5). Equally apparent were large numbers of smooth membrane-bound vacuoles (approximately 0.2 μm in diameter) dispersed in several regions of the cells (Fig. 6). Several of the larger Golgi vacuoles contained parallel aggregates of filamentous structures approximately 6,000 Å in length (Fig. 7). In Fig. 7 one of these can be seen to contain a region of increased density in the form of a line in the center of the aggregate. Microtubules,

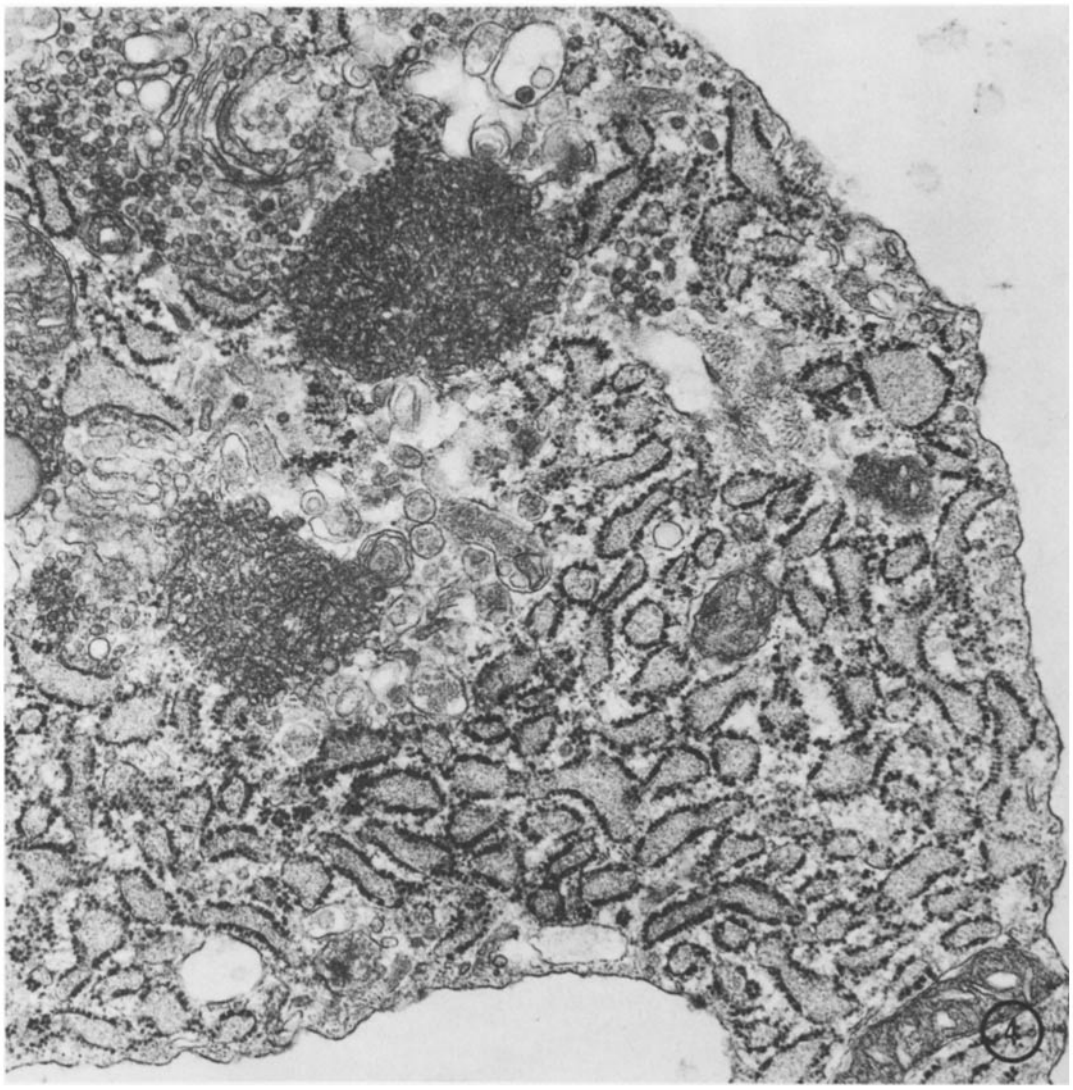

FIGURE 4 This electron micrograph is a higher magnification of a segment of the cell seen in Fig. 3. The membranous aggregates present in this cell differ from the crystalloid structures previously reported in cells treated with vinblastine. The vesiculated appearance of the rough endoplasmic reticulum is more readily apparent at this increased magnification. × 29,000.

which were readily apparent in control cells, were absent from colchicine-treated preparations. The filaments seen adjacent to a mitochondrion on the left side of Fig. 6 represent 100-Å microfilaments.

As noted previously, α,α'dipyridyl, an iron chelator, inhibits peptidyl hydroxylation and collagen secretion. Electron microscope examination of α,α'dipyridyl-treated bone revealed that the cells were relatively normal in appearance. The Golgi complex was not distended, and unlike the situation in fibroblasts from scorbutic animals, the configuration of the RER cisternae appeared to be unaltered (Fig. 8). However, examination of the configuration of ribosomal aggregates attached to the RER membrane showed that in many of the cells the characteristic configuration had been lost. Thus, tangential sections of the RER membranes providing an *en face* view of the ribosomal pattern demonstrated that the attached ribosomes appeared to be randomly distributed on the surface of

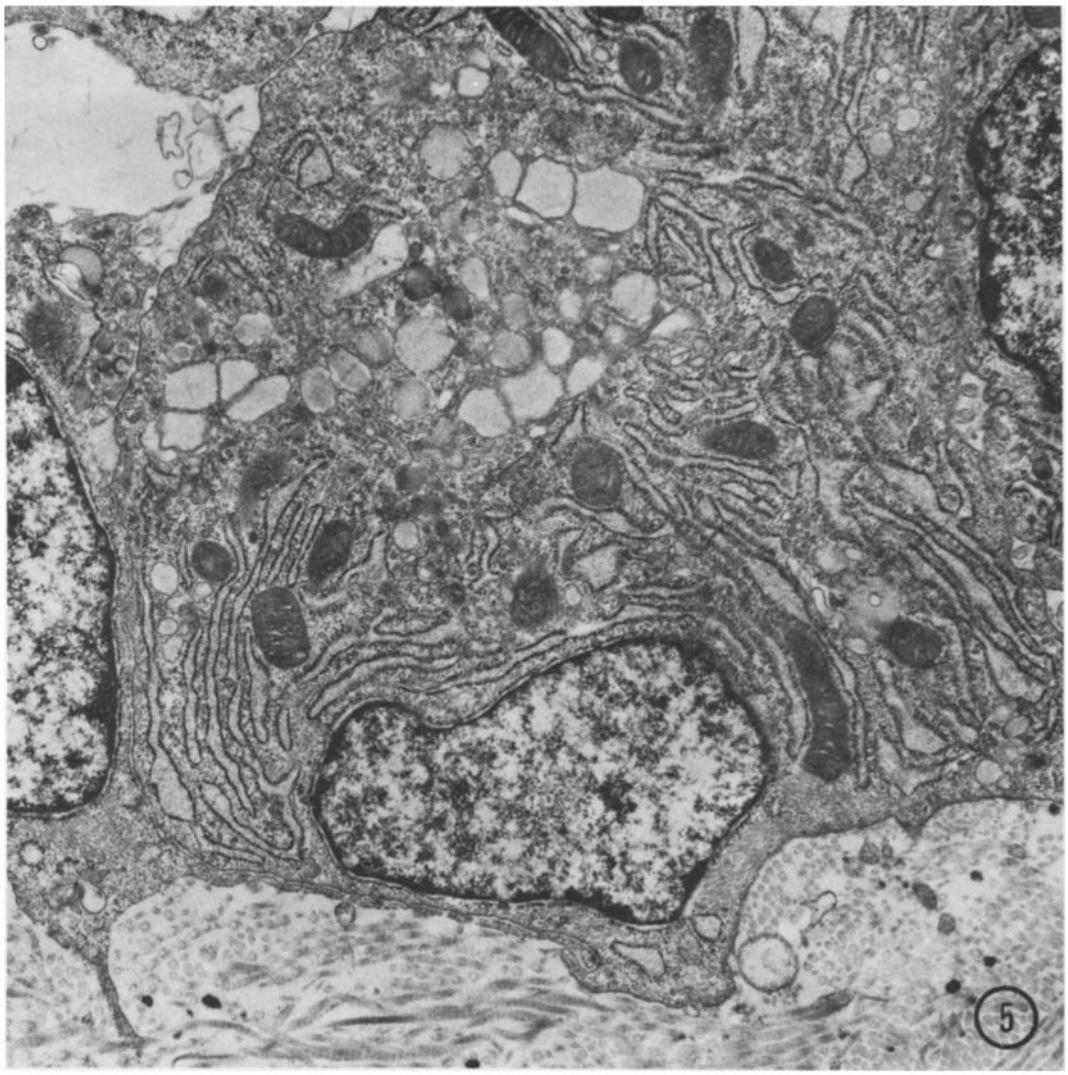

FIGURE 5 This is a relatively low-power electron micrograph of several osteoblasts in bone treated with colchicine. The marked enlargement of Golgi-associated vacuoles can be seen. The rough endoplasmic reticulum in this cell is not altered in its appearance. In a number of the Golgi vacuoles filamentous structures are seen which are shown at higher magnification in Fig. 7. The appearance of increased numbers of large Golgi-associated vacuoles was the most striking finding after colchicine treatment. × 14,000.

the membrane (not shown). This observation is similar to one made earlier on ascorbic acid deficiency (40).

The cells treated with cytochalasin B differed in their ultrastructural appearance in that peripheral 60-Å filaments were visible in some cells but not in others; the larger cytoplasmic filaments (100 A) were visible in all cells. Some cells contained dilated cisternae of RER but this was also variable from cell to cell.

DISCUSSION

Antimicrotubular agents such as vinblastine and colchicine have been used to implicate microtubules in colloid droplet formation and hormone secretion by the thyroid gland (51, 52), and in the secretory activity of a number of other cell types (14, 15, 28, 50). However, not all secretory processes involving particulate material are inhibited by these substances, and in some instances the

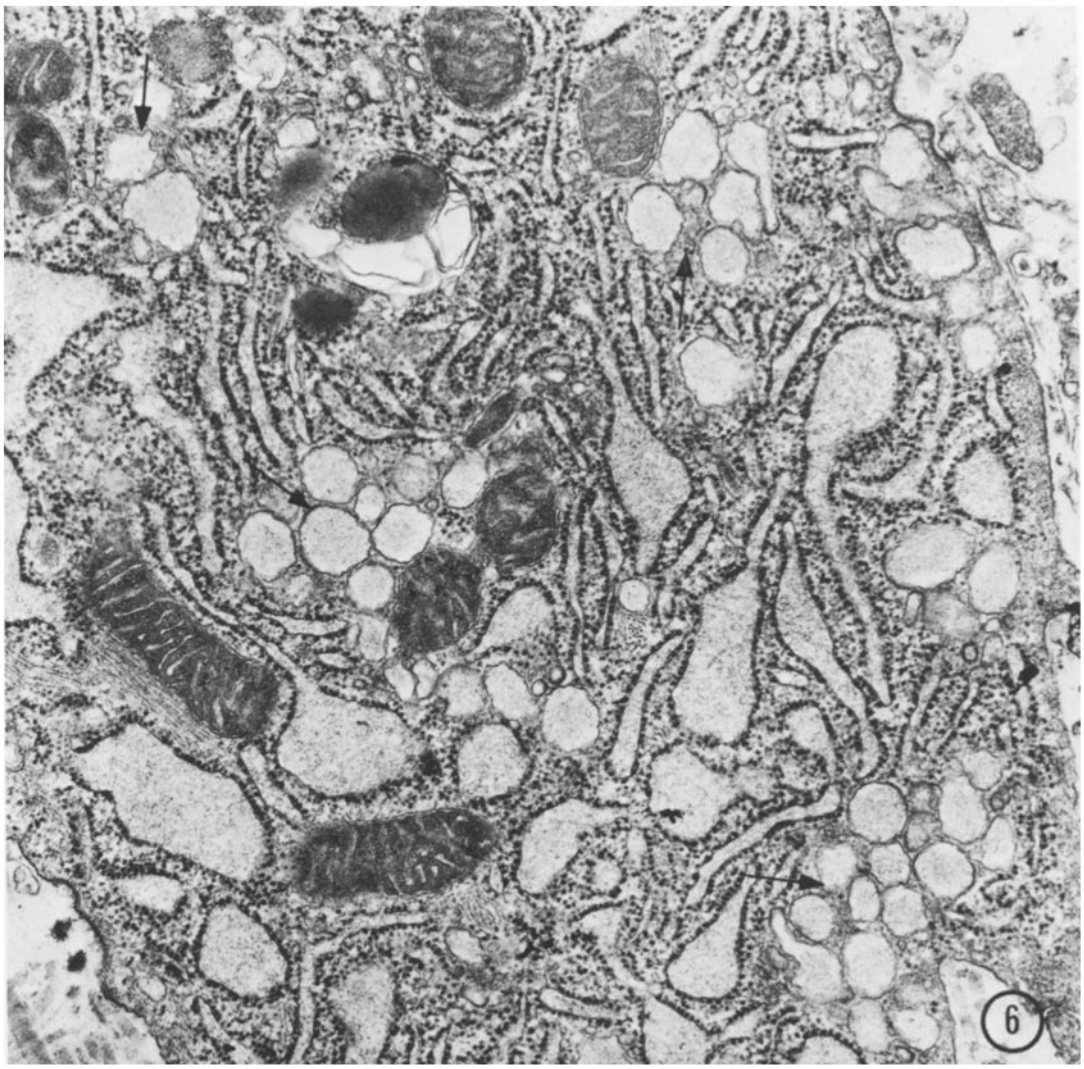

FIGURE 6 This electron micrograph demonstrates part of an osteoblast from colchicine-treated bone. The characteristic appearance of increased numbers of enlarged Golgi-associated vacuoles can be seen. There are at least four foci of enlarged Golgi vacuoles (arrows) present. The rough endoplasmic reticulum of this cell demonstrates the usual three-dimensional canalicular array characteristic of this organelle. × 29,000.

effects may be unrelated to disruption of microtubular function. Thus, colchicine and vinblastine were reported to stimulate the release of glucagon by isolated guinea pig islet cells (11). In the case of catecholamine release by adrenal glands, the inhibition observed with colchicine and vinblastine (34) has been attributed to an anticholinergic action rather than a generalized effect on microtubules (45).

Colchicine was shown to be capable in organ culture of inhibiting the resorption of bone induced by parathyroid hormone or by metabolites of vitamin D (36). An inhibitory effect on osteoclastic activity was postulated. In cultures of synovial tissue, colchicine increased the secretion of collagenases into the medium (19). The basis for these effects is not understood.

Consideration of the actions of antimicrotubular agents is further complicated by well documented metabolic effects apparently unrelated to changes in the cytoskeleton. The transmembrane movement of several nucleosides was shown to be

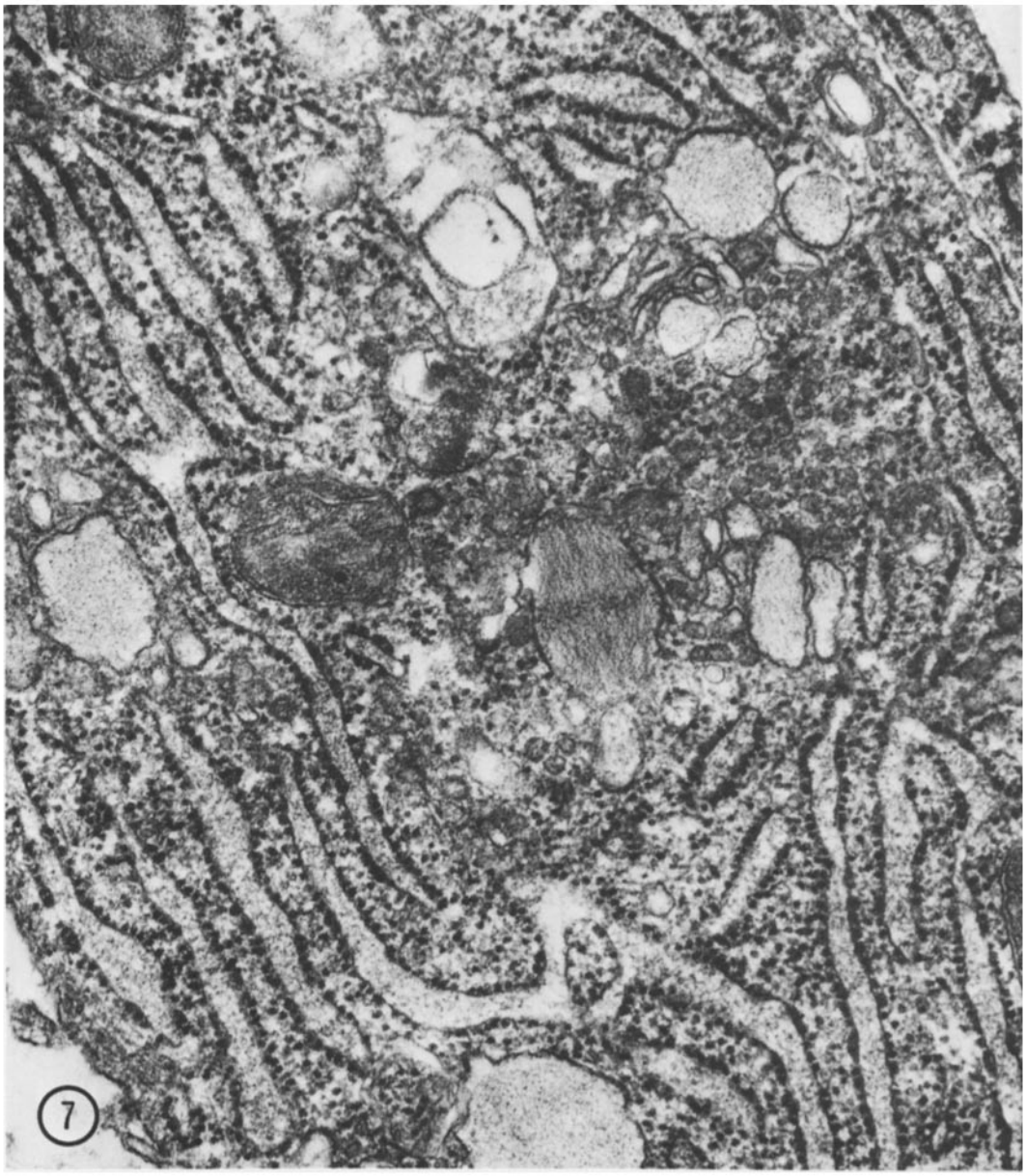

FIGURE 7 This electron micrograph demonstrates part of an osteoblast from colchicine-treated bone in which one of the enlarged Golgi-associated vacuoles contains an aggregate of filaments with a dense line appearing in the center. The overall length of these aggregates is approximately 6,000 Å with the distance from the center to the end being approximately 3,000 Å. Similar aggregates can be seen in Golgi vacuoles in Fig. 5. × 55,000.

inhibited by colchicine in a number of mammalian cell lines (30), and this compound was also shown, albeit in high concentrations, to inhibit glucose-6-phosphate and 6-phosphogluconate dehydrogenases leading to an inhibition of the hexose monophosphate pathway in leukocytes (8).

With these reservations in mind we have analyzed the effects of antimicrotubular agents at the

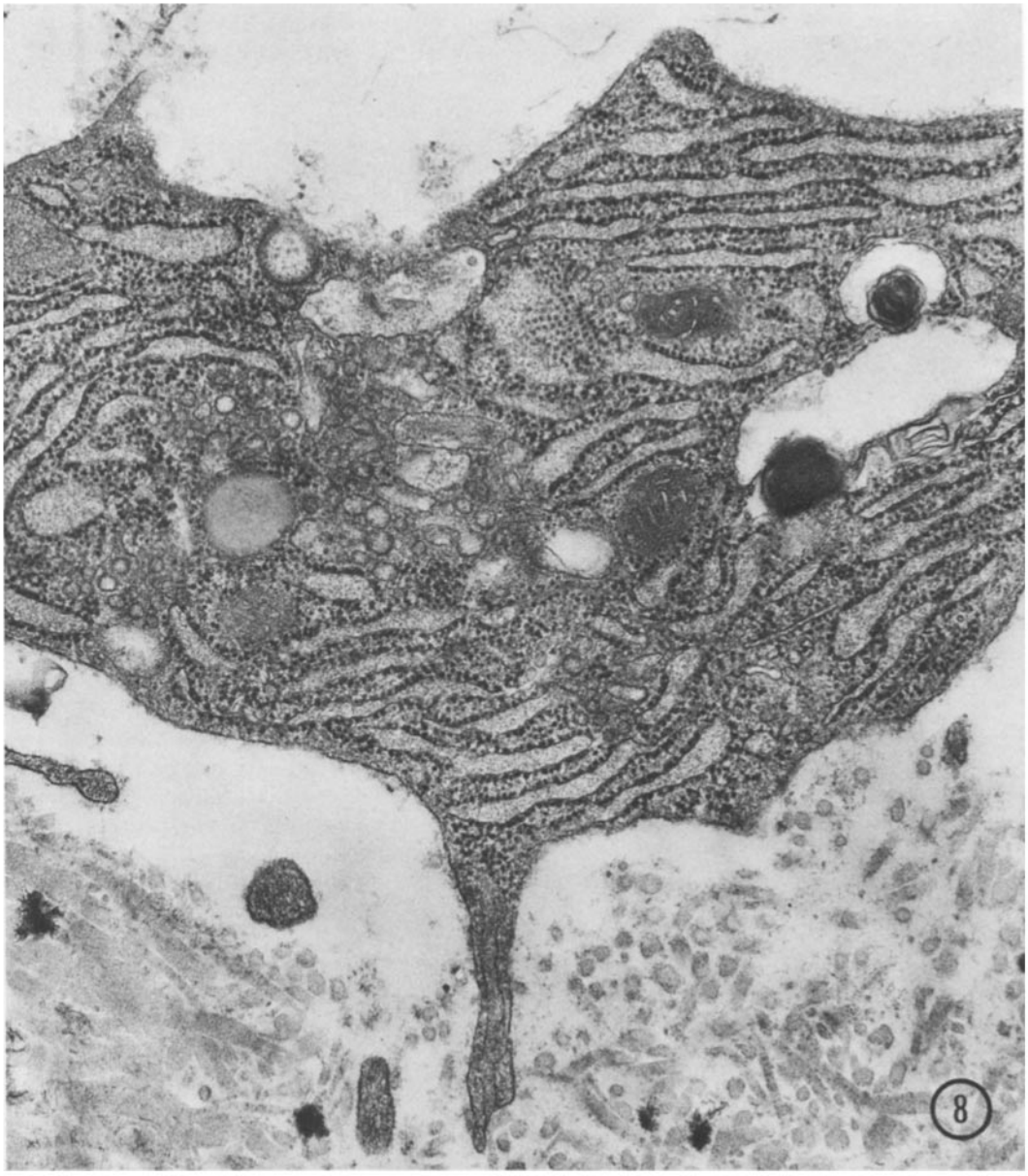

FIGURE 8 In this electron micrograph of an α,α'dipyridyl-treated osteoblast, the three-dimensional canalicular distribution of the endoplasmic reticulum cisternae can be seen to be usual in appearance. The Golgi complex is not markedly enlarged and the cell does not appear to be otherwise altered. × 30,000.

ultrastructural level and on protein synthesis by cranial bone in culture. An attempt was made to interpret the results in terms of a mechanism for the intracellular translocation and secretion of collagen.

Colchicine clearly retarded the conversion of procollagen to collagen and inhibited the secretion of proline-labeled material as judged by light microscope autoradiography (Table I). Since the vast majority of the protein secreted by cranial bone cells in the 17-day old chick embryo is collagen, and since the imino acid content of

collagen is more than five times that of noncollagenous proteins, the distribution of silver grains in this tissue can be equated with that of collagen. The lack of effect of colchicine on noncollagen-protein synthesis observed in this study (Fig. 2, Table III) is supported by previous studies on chick cranial bone (12), fetal rat long bone (36), and embryonic lens epithelium (32). Consistent with these findings was the observation that colchicine produced no morphologic alterations in the RER. However, the Golgi complex contained a marked increase in Golgi-associated vacuoles and vesicles, some of which contained aggregates of filamentous structures similar to those reported in other studies (48, 49). The dimensions of some of these structures (6,000 Å, seen in Fig. 7) are consistent with their identification as parallel bundles of collagen aligned and stacked end to end.

These observations suggest that at least a part of the collagen secreted by the cells is routed through the Golgi complex, and they further imply that microtubules normally function in the transcellular movement of Golgi-derived vesicles. It is not clear whether the microtubules provide pathways for intracellular movement of vesicles or whether they actually assist in the movement as well. The gradual inhibition of collagen synthesis by colchicine (Fig. 2) may be attributed to disruption of the Golgi pathway. Since there is now good evidence that at least the final step in the conversion of procollagen to collagen occurs extracellularly (3, 27, 42), retention of procollagen within the Golgi complex would be expected to interfere with procollagen conversion. Work recently completed in our laboratory (to be submitted for publication) provides support, based on immunological localization, for the role of the Golgi complex in the secretion of collagen.

However, alternate secretory pathways functioning simultaneously in the cell cannot be excluded by these findings. If a fraction of the collagen bypasses the Golgi complex and is secreted by intermittent communication of cisternal elements with the extracellular space, interruption of this process may not result in changes in the RER which are evident morphologically. Furthermore, movement of such cisternal elements may not be dependent on microtubules, or colchicine may not interfere with RER-associated microtubular function.

The effects of vinblastine on cranial bone cells at the concentrations used in this study are more complex, as indicated by an inhibition of both collagen and noncollagen-protein synthesis (Fig. 1, Tables II and III). Vinblastine, at concentrations of 10^{-5} M, has been reported to produce complexes between polyribosomes and the crystals induced by the drug in L cell fibroblasts and lymphoblasts (26). Such complexes may well interfere with protein synthesis and lead to an inhibition in incorporation of amino acids as was observed in this study. Ultrastructurally, the vacuolar appearance of the RER in vinblastine-treated preparations (Fig. 3 and 4) contrasted with the normal interconnecting canalicular structure of this organelle in colchicine-treated or untreated osteoblasts. The lack of stored material in the Golgi complex after vinblastine treatment, despite disruption of microtubules, may reflect concurrent inhibition of protein synthesis. It is of interest that the appearance of RER cisternae after exposure to vinblastine is strikingly similar to changes previously observed in ascorbic acid deficiency in vivo (40) in which collagen synthesis is also inhibited.

The effects of cytochalasin B on the synthesis and secretion of collagen by cranial bone cells have been difficult to evaluate. Light microscope autoradiographic examination revealed that the secretion of collagen by some cells in bones exposed to cytochalasin B was markedly inhibited, whereas other cells in close proximity were apparently unaffected. As a result the significance of the quantitative difference observed in the proportion of grains retained intracellularly after cytochalasin B treatment (Table I) is unclear. Ultrastructurally, a similar variability was seen in the appearance of both osteoblasts and fibroblasts treated with cytochalasin B. Heterogeneity in the response of baby hamster kidney cells to cytochalasin B, with respect to changes in cell shape and tendency to enucleate, has also been reported (17). Possibly both the configuration of intracellular microfilaments and the susceptibility to the drug may vary with different stages of the cell division cycle.

No inhibition of incorporation of leucine was found after a relatively short incubation of cells in the presence of cytochalasin B (35, 41), although Yamada et al. (53) observed a 20% reduction in protein synthesis by dorsal root ganglia over a 2-h period using a mixture of labeled amino acids. In a previous study (12) cytochalasin B (5 μg/ml) was found to have no effect on tryptophan incorporation into protein extracted from chick cranial bone with 0.5 M acetic acid and 0.1% Triton X-100.

However, the present results, which reflect total tissue protein rather than an extractable fraction, show relatively equal inhibition of both collagen and noncollagen-protein synthesis by cytochalasin B (Fig. 1, Tables II and III). In view of this effect, the variability of the cellular response to cytochalasin B at the light microscope level, and the lack of consistent changes in the ultrastructure of the RER and Golgi complex, conclusions relevant to the mechanism of collagen secretion cannot be drawn from our use of this compound.

In conclusion, both microtubule inhibitors, vinblastine and colchicine, decrease collagen synthesis and secretion but in so doing lead to different ultrastructural changes in cells. The effects of vinblastine are manifest in both the RER and the Golgi complex, resulting in a vesiculation of the RER cisternae and a diminution of the Golgi complex. Colchicine appears to affect only the Golgi complex, leading to enlarged Golgi-associated vacuoles. The changes seen with vinblastine are consistent with the generalized depression of protein synthesis produced by the compound. The more limited changes in the Golgi complex seen with colchicine were associated with a 30% reduction in collagen synthesis and with the appearance of abnormal filamentous structures within Golgi-associated vacuoles. The latter findings suggest that collagen is secreted at least in part via the Golgi pathway and that this secretion is dependent on a normally functioning microtubular system.

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