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Intracellular processing of procollagen induced by the action of colchicine

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INTRODUCTION

Dehm & Prockop (1972), Diegelmann & Peterkofsky (1972) and Ehrlich & Bornstein (1972) have shown that colchicine produces an intracellular accumulation of collagen by markedly inhibiting its secretion. This agent was found to retard the conversion of procollagen to collagen in cultured cranial bones (Ehrlich & Bornstein, 1972). Subsequently, it was reported that cells exposed to colchicine showed an increased number of Golgi-derived vacuoles, some of which contained parallel aggregates of filamentous structures which were assumed to represent intracellular procollagen (Ehrlich, Ross & Bornstein, 1974; Olsen & Prockop, 1974; Scherft & Heersche, 1975). Several electron microscopical studies of collagen-producing cells have identified smooth-surfaced vacuoles, apparently derived from the Golgi complex, and containing fibrillary material which may be collagenous in nature (Hay & Dodson, 1973; Trelstad, 1971; Weinstock, 1972; Weinstock & Leblond, 1974).

In view of this evidence that colchicine retards the secretion of collagen precursors, we felt that this agent would be useful in the identification of these macromolecules within cytoplasmic vacuoles. In this work we report the massive induction of intracellular segment long spacing crystallites by colchicine. A preliminary account of our work has been published previously (Fernandez-Madrid, Noonan & Riddle, 1974).

ABBREVIATIONS AND TERMINOLOGY

The following abbreviations have been used in this paper: SLS, segment long spacing; TC, tropocollagen; SDS, sodium dodecyl sulfate; NEM, *n*-ethylmaleimide; DFP, diisopropyl fluorophosphate; EDTA, ethylenediamine tetraacetic acid; PMSF, phenyl methyl sulphonyl fluoride; MEM, Earle's minimal essential medium; BAPN, β-aminoproprionitrile; N, amino; C, carboxyl. We use the term procollagen for any precursor of collagen with polypeptide chains larger than those of tropocollagen.

MATERIALS AND METHODS

Biochemical procedures

Eggs from white Leghorn chickens obtained from Holtzapples Hatcheries (Romeo, MI.) were incubated in a moist atmosphere at 37 °C until used in our studies. All

experiments were performed using chick embryos that were 11 days old. [^{3}H]proline (43·6 Ci/m-mole) was purchased from New England Nuclear Corp., Boston, Mass. Colchicine was obtained from Eli Lilly and Co. Indianapolis, Indiana. β-mercaptoethanol and ammonium persulphate were purchased from Baker Chemical Company, Phillipsburg, New Jersey. Sodium dodecyl sulphate (SDS), *N*-ethylmaleimide (NEM), diisopropyl fluorophosphate (DFP), ethylenediamine tetraacetic acid (EDTA), phenyl methylsulphonyl fluoride (PMSF), and benzamidine hydrochloride were obtained from Sigma Chemical Co., St Louis, Mo. The source of collagenase type II was Advanced Biofactors, Inc., Lynbrook, New York, whereas, *N,N'*-methylenebisacrylamide, acrylamide and *N,N,N',N'*-tetra methylethylenediamine (electrophoresis grade) were obtained from Eastman, Rochester, New York. The scintillant employed was Aquasol-2, purchased from New England Nuclear, Boston, Massachusetts. All glassware used in handling reduced proalpha chains was siliconized with sigmacote (Sigma).

Corium fragments were prepared from chick embryos 11 days old by the method of Noble & Boucek (1965). They were collected at room temperature in Earle's minimal essential medium (MEM) with the addition of 8 units/ml of penicillin and 8 μg/ml of streptomycin. Two groups were separately collected and at the end of the collection the corium fragments were washed once with fresh MEM. To both groups were added MEM, penicillin (8 units/ml), streptomycin (8 μg/ml), β-amino proprionitrile (BAPN) 64 μg/ml and 100 μg/ml Na ascorbate. The total volume in both groups was 20 ml. To one of these groups varying concentrations of colchicine were added. Since it was found that 2·5 μM/ml colchicine consistently inhibited the secretion of procollagen *in vitro*, all subsequent experiments were done using this concentration to avoid the cellular damage observed at higher doses. The incubation was terminated after 3 hours by placing the corium fragments in 0·6 M cacodylate-buffered glutaraldehyde (1 %, w/v) and processing them for electron microscopical studies. For the *in vivo* experiments, viable chick embryos 11 days old were injected in the yolk sac with varying concentrations (0, 1, 2·5, 6·5 and 12·5 μM) of colchicine in 0·2 ml of 0·9 % NaCl. Injections were performed with glass microneedles through a window in the shell which was closed and sealed with paraffin, and *in vivo* incubation was continued at 37 °C. The embryos were killed after 3 hours, and the corium fragments were immediately fixed for ultrastructural studies as before.

Radioactivity incorporation and preparation of procollagen

For the studies of the incorporation of radioactivity into collagen precursors, after incubating the diced corium fragments in a Dubnoff shaker as described above in either the presence or absence of colchicine for two hours at 37 °C, 20 μCi [^{3}H]proline/ml were added to each of the groups, and incubation was continued for 60 minutes. The incubations were terminated by the addition of 10 ml of ice-cold MEM solution, and the corium fragments were collected by centrifugation at 500 g for 10 minutes. Procollagen was partially purified from the tissue and media by successive precipitation with NaCl and Cl_3C COOH (Monson, Click & Bornstein, 1975). The tissue was extracted by rapid homogenization in a glass tissue homogenizer in extraction buffer containing 1 M-NaCl, 0·5 M tris-HCl (pH 7·5), 0·025 M EDTA, 0·01 M NEM, 0·001 M DFP and 0·001 M benzamidine-HCl. The mixture was allowed to stand overnight. Solid NaCl was added to a concentration of 20 %, stirred for 1 hour, and centrifuged for 20 minutes at 37000 g. The small precipitate was re-suspended in 20 ml of extraction buffer. The extracted homogenate was

centrifuged for 20 minutes at 36000 g at 4 °C. NaCl was slowly added to the supernatant to a final concentration of 20 % and stirred for 1 hour. The precipitate was collected by centrifugation for 1 hour at 36000 g at 4 °C and was re-suspended and extracted overnight in 20 ml of buffer. The extract was centrifuged for 20 minutes at 36000 g at 4 °C and the clarified extract was precipitated by adding 40 % trichloroacetic acid (TCA) to bring the sample to 5 % TCA. The precipitate was re-suspended in 10 ml extraction buffer, dialysed against water with the addition of 0·01 M NEM, 0·01 M PMSF, 0·025 M EDTA and 0·001 M benzamidine hydrochloride. After three changes, the radioactivity was determined in aliquots as previously described (Fernandez-Madrid, 1967). The remainder of the extracts were lyophilized and, prior to electrophoresis, the samples were re-suspended in 0·01 M-Na phosphate, 0·1 % SDS, 0·5 M urea and 1 % β-mercaptoethanol. These samples were heated at 55 °C for 1 hour. Analytical gel electrophoresis of procollagen and collagen chains labelled with [^{3}H]proline was performed in 5 % polyacrylamide gels that contained 0·1 % SDS in running buffer (0·1 M-Na phosphate, 0·1 % SDS and 0·5 M urea and 0·005 M β-mercaptoethanol) for 4·5 hours at 12 mA/13 cm gel (Goldberg, Epstein & Sherr, 1972). From 61 to 92 % of the radio-labelled material applied to the gels was accounted for when the gel slices were counted. Heat denatured, salt extracted rat tail tendon collagen served as an internal standard to mark the position of migration of α and β chains which were determined by staining with Coomassie blue, and destained electrolytically in 7 % acetic acid and 5 % methanol (Webber & Osborn, 1969). Unstained gels were cut into 1 mm slices, which were dissolved with 0·2 ml hydrogen peroxide, incubated overnight at 56 °C and then cooled and counted in 10 ml Aquasol-2 as before. Collagenase digestion with purified bacterial collagenase (Advanced Biofactors) was performed as described by Peterkofsky & Diegelmann (1971).

Control preparations

Chick embryos 11 days old were injected *in vivo* with 0·2 ml of saline, the vehicle used to inject the colchicine. They were killed at identical intervals and handled in the same way as those treated with colchicine. For the *in vitro* experiments, chick embryo corium fragments were incubated under identical conditions except that colchicine was omitted.

Ultrastructural procedures

The corium fragments from both *in vivo* and *in vitro* experiments were processed identically for electron microscopy. They were immediately placed in 0·6 M cacodylate-buffered (1 %, w/v) glutaraldehyde (pH 7·2–7·4) and allowed to fix for 24 hours at 4 °C (Sabatini, Miller & Barnett, 1964). Following fixation the samples were washed overnight with 0·2 M cacodylate-buffered sucrose (pH 7·2–7·4), post-fixed and stained for 2 hours in 1 % veronal acetate buffered osmium tetroxide (pH 7·2–7·4), progressively dehydrated with ethanol, and embedded in Maraglas (Freeman & Spurlock, 1962). Ultrathin sections, 20–40 nm thick, were obtained using a Porter-Blum MT-1 ultramicrotome equipped with a diamond knife. The sections were mounted on unsupported 300 mesh copper grids and doubly stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Examination and photography were performed with an RCA EMU-4 transmission electron microscope operated at 50 kV.

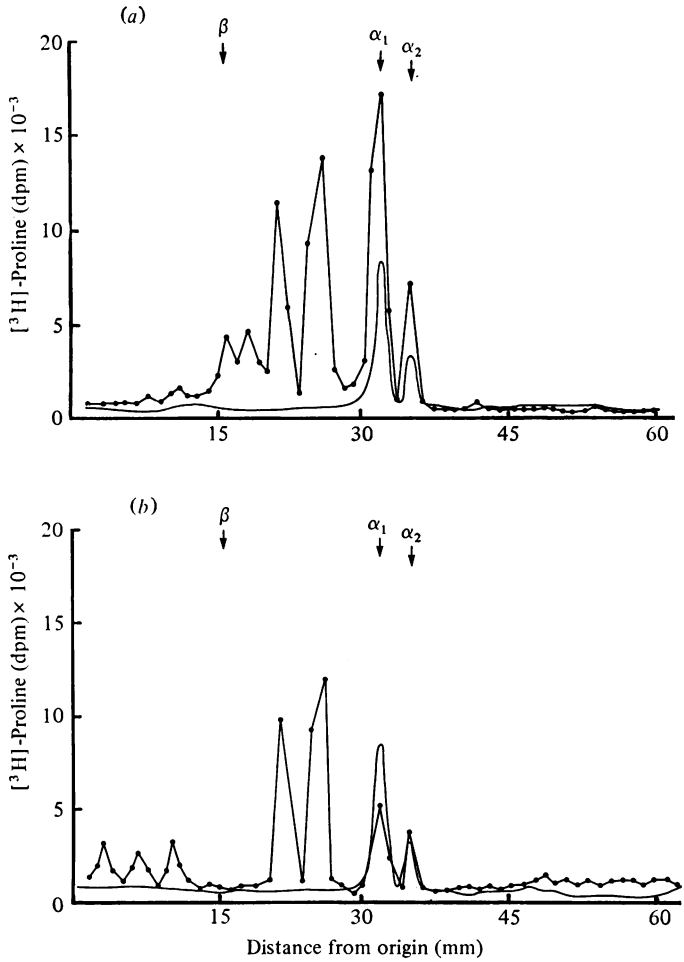


Fig. 1. Sodium dodecyl sulphate-urea-polyacrylamide gels of extracts from chick embryo corium. The plots show the distribution of radioactivities between treated and untreated preparations. ——, Carrier rat tail tendon collagen; ●—●, [^{3}H]proline. (*a*) Control; (*b*) 2·5 μM colchicine.

RESULTS

Electrophoresis of collagenous products

Corium fragments obtained from 15 dozen embryos were incubated as described above in the presence or absence of 2·5 μM colchicine for 2 hours. Then 20 μCi [^{3}H]proline/ml were added to each vessel and incubation was continued for 1 hour. From each of the incubation mixtures procollagen and collagen chains were partially purified by successive precipitation with NaCl and Cl_3CCOOH in the presence of inhibitors of proteases as described by Monson, Click & Bornstein (1975). The extracts were lyophilized and re-suspended in buffer containing 0·01 M-Na phosphate, 0·1 % SDS, 0·5 M urea and 1 % β-mercaptoethanol, and then denatured by heating at 55 °C for 1 hour prior to electrophoresis in 5 % polyacrylamide gels. The ratio of the dpm in procollagen and collagen chains was determined by integrating the dpm (minus background) in the appropriate fractions. No attempts were made to

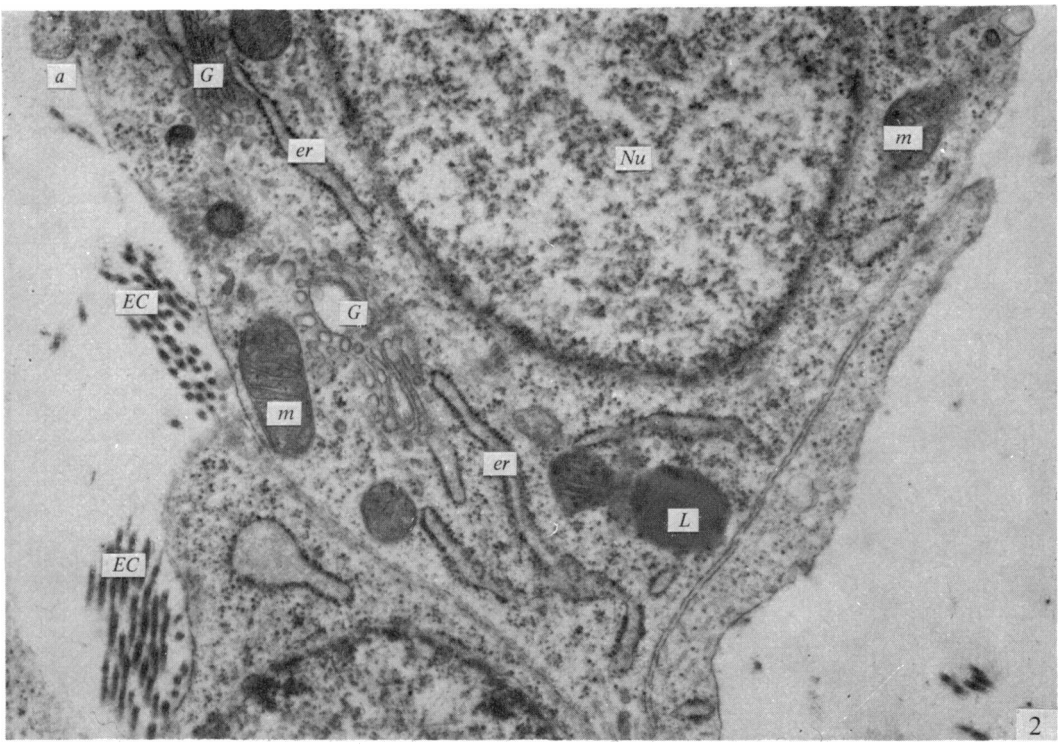


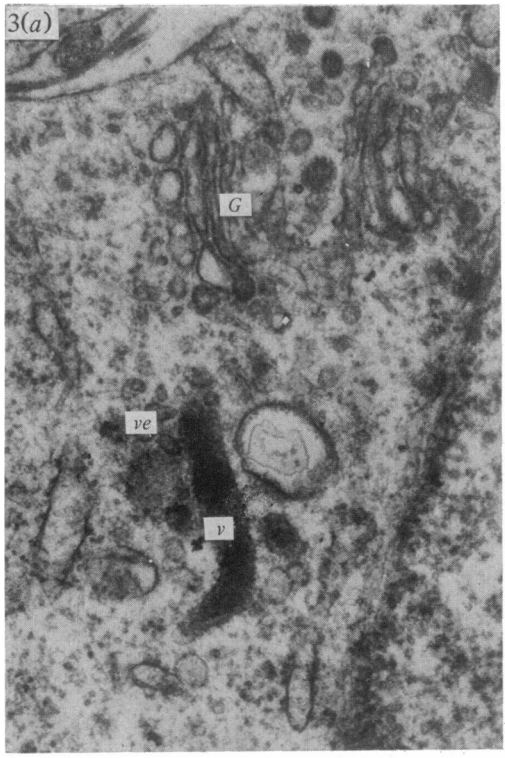
Fig. 2. Control fibroblasts 3 hours after *in vivo* injections of 0·2 ml saline (0·9 %). Typical ultrastructure of control chicken embryo corium fibroblasts with large nuclei (*Nu*), mitochondria (*m*), rough endoplasmic reticulum (*er*), Golgi complexes (*G*) and an occasional lipid inclusion (*L*). Extracellular collagen (*EC*) was usually present close to the plasma membrane. × 18000.

determine specific precursors and products. The ratio of the radioactivities incorporated with procollagen and collagen chains therefore reflects the differences in the proportions of the sum of the precursors and the sum of the processed chains.

SDS acrylamide gel electrophoresis of the reduced collagenous products isolated from untreated chick embryo corium showed two major procollagen peaks migrating behind the alpha chains (Fig. 1*a*). In the preparation pre-treated with 2·5 μM colchicine, there was an increase in the proportion of procollagen with respect to alpha chains (Fig. 1*b*) which is reflected in the higher procollagen/collagen ratio in the colchicine-treated preparation (2·64) when compared to that of the controls (1·01). Pre-treatment of the preparations with highly purified bacterial collagenase degraded completely the procollagen peaks as well as the alpha chains (not shown). Two identical experiments gave similar results.

Ultrastructural observations

Fibroblasts from control preparations examined 3 hours following an intravenous injection of 0·9 % saline (drug vehicle) appeared morphologically identical to untreated chick embryo corium fibroblasts. The fine structure of these fibroblasts revealed large irregularly shaped nuclei, a cytoplasm containing scattered mitochondria with distinct cristae, many profiles of rough endoplasmic reticulum (RER), an occasional Golgi complex, microtubules and fine non-striated filaments (Fig. 2).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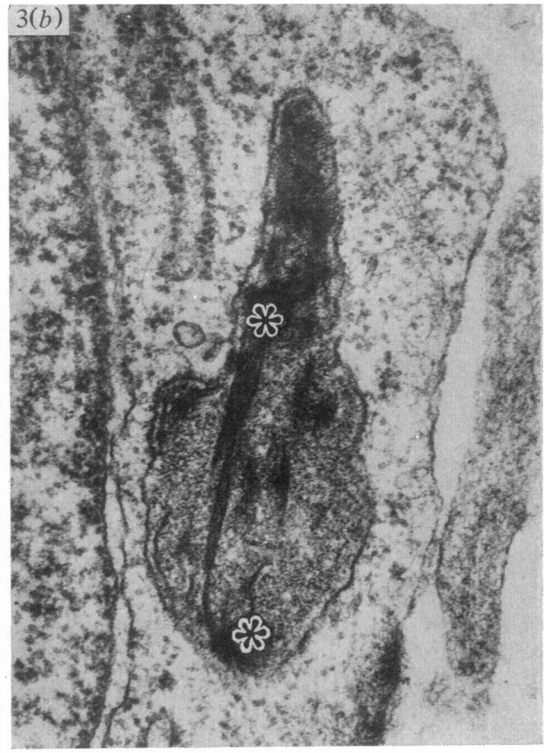
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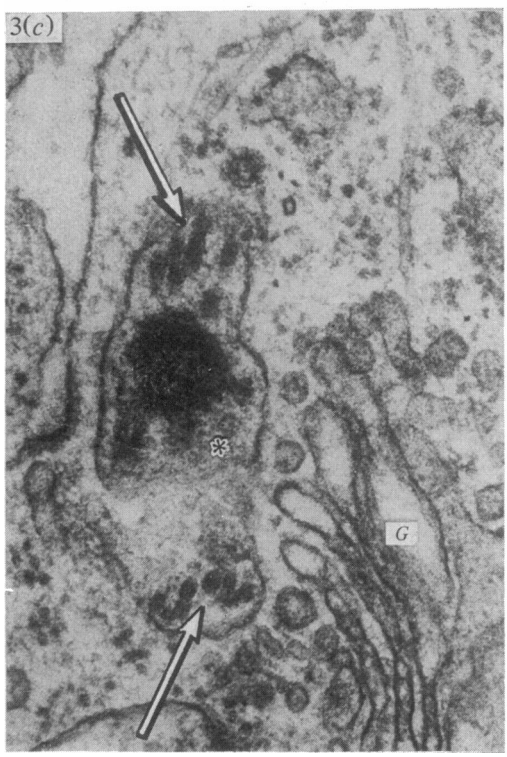
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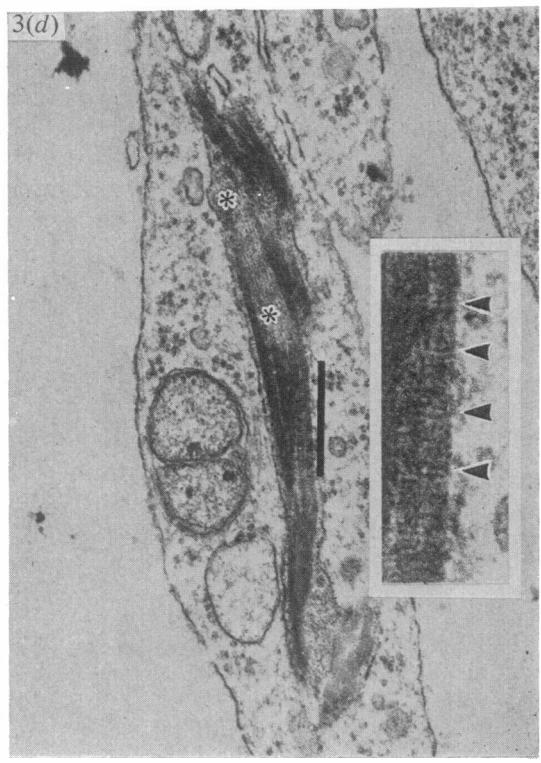
3(d)

The ultrastructure of *in vivo* and *in vitro* chick embryo corium treated with colchicine was essentially identical to that of control preparations except for the increased number of fibroblasts containing a spectrum of membrane-limited vacuoles which contained electron-dense material in various concentrations. Vacuoles were observed which contained only amorphous material (Fig. 3*a*), while other vacuoles revealed admixtures of structural organizations embedded within the amorphous material (Fig. 3*b–d*). The intra-vacuolar material appeared to condense into filamentous arrangements (Fig. 3*b*). The increasing formation of these filamentous arrangements appeared to distend the vacuoles into very elongated shapes (Fig. 3*b–d*). Transition between an amorphous electron-dense substance and non-striated fibrils (Fig. 3*b*), and between non-striated fibrils and fibres with periodicity (Fig. 3*d* and inset), could be observed within some of the vacuoles. The measurement of these fibres yielded period of 50–55 nm (Fig. 3*d*), approaching those of native extracellular collagen. Many of these vacuoles contained fibrillar aggregates affecting the form of segment long spacing crystallites which were embedded in an electron-dense amorphous material (Figs. 4, 5). The SLS found in these cytoplasmic vacuoles presented an arithmetic mean of 230 nm in length and a band pattern distinctly polarized (Fig. 5). A comparison of the intracellular SLS crystallites with crystallites reported in the literature shows that both ends of the triple helical portion of the molecule were intact and that no additional bands were present at the amino or carboxy ends (Fig. 5).

Intracytoplasmic vacuoles were frequently observed in juxtaposition to Golgi complexes (Fig. 3*a*, *c*). Small vesicles similar to those derived from the Golgi apparatus were seen in close proximity to the vacuolar membrane (Fig. 3*a*, *c*) as well as randomly intermixed with the amorphous matrix contained within the intravacuolar compartment (Fig. 3*c*).

DISCUSSION

Several studies have suggested that cytoplasmic vacuoles probably related to the Golgi complex observed in collagen-producing cells are involved in the secretion of collagen precursors (Frank, 1970; Harwood, Grant & Jackson, 1973; Hay & Dodson, 1973; Nist *et al.* 1975; Olsen & Prockop, 1974; Revel & Hay, 1963; Trelstad, 1971). Small vesicles containing parallel arrays of non-striated filaments were also described by Weinstock (1972) and by Weinstock & Leblond (1974) in odontoblasts, and by Ehrlich, Ross & Bornstein (1974) and by Olsen & Prockop (1974) in colchicine-treated cells. Colchicine binds with microtubules and interferes with their assembly (Borisy & Taylor, 1967), and this effect has been thought to be the basis of the inhibitory action of the alkaloid on various types of secretions (Lacy, Howell, Young & Fink, 1968; Williams & Wolff, 1970). It has also been shown that colchicine can be bound by membranous fractions (Feit & Barondes, 1970) and that it can affect

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Fig. 3. Fibroblasts treated with 2·5 μM colchicine *in vivo* demonstrated a variety of cytoplasmic membrane-limited vacuoles. (*a*) Vacuole (*v*) containing amorphous electron-dense material, surrounded by vesicles (*ve*), and in close juxtaposition to a Golgi complex (*G*): ×38000. (*b*) Cytoplasmic vacuole containing non-striated fibrils (*) embedded in an amorphous matrix: ×44000. (*c*) An elongated vacuole containing amorphous material, fibre formations (arrows), and small vesicles (*) close to a Golgi complex (*G*): ×66000. (*d*) a vacuole containing non-striated fibrils (*) in transition with fibres demonstrating periodicity (bar): ×41000. Inset contains the membrane-bound fibre in (*d*) (bar) showing an average periodicity of 50 nm. ×120000.

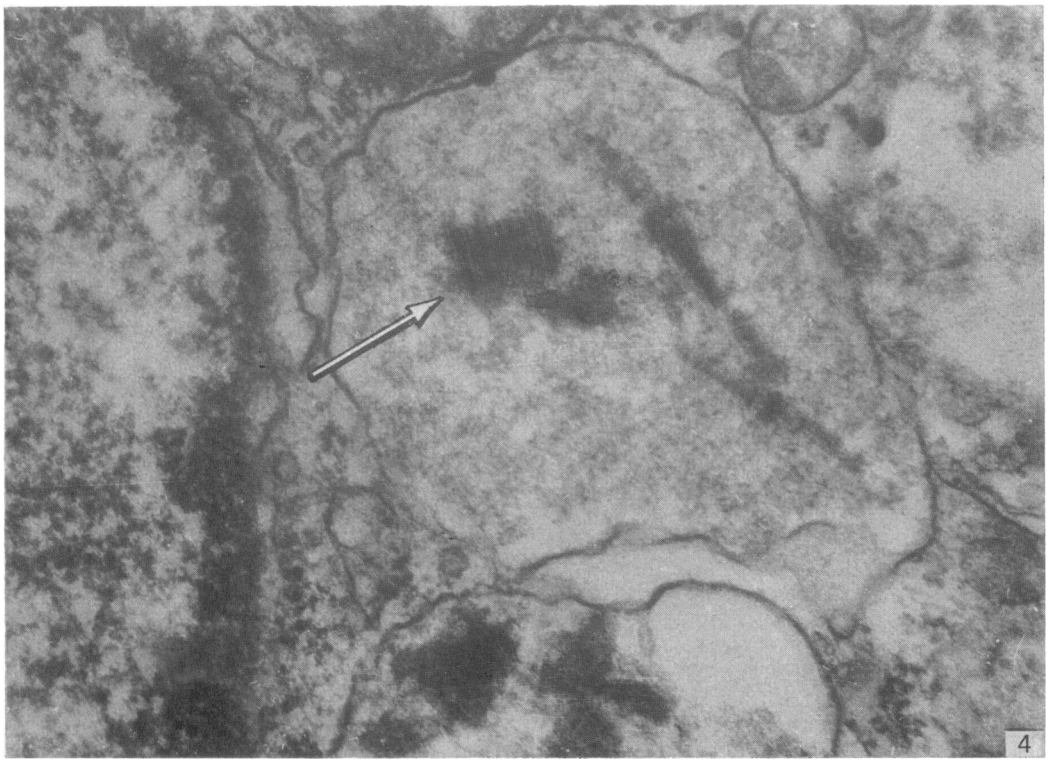

Fig. 4. A colchicine-treated fibroblast (2·5 μM, *in vivo*) with a vacuole containing segment long spacing (*SLS*) crystallites (arrow), embedded in an electron-dense amorphous material. × 75500.

the properties of membrane components (Vasiliev *et al.* 1970). These and other observations (Hindelang–Gertner, Stoeckel, Porte & Sutinsky, 1976) suggest a complex mechanism of action of colchicine on cytoplasmic movements and secretion. As part of the morphological spectrum produced by the action of colchicine we have observed vacuoles containing fibrous aggregates which can be recognized as collagenous because of their periodicity (Figs. 3*d*, 4, 5). The feature which permits the identification of collagen with the electron microscope is of course its repeating periodic structure (Schmitt, Hall & Jakus, 1942). The presence of tropocollagen in the vacuoles found in our work is suggested by the findings of SLS crystallites (Figs. 4, 5). A common macromolecular species, tropocollagen, was deduced from the reversible interconversion of SLS, fibrous long spacing (FLS), non-striated fibrils and fibrils with native periodicity, from solutions of collagen, adjusting the usual environmental parameters such as pH and ionic strength (Schmitt, Gross & Highberger, 1953). A similar phenomenon may occur within the vacuoles formed in the fibroblasts treated with colchicine. SLS obtained from positively stained preparations have been found to be approximately 10 % shorter than those obtained with negative staining (Cox, Grant & Horne, 1967). In addition to being positively stained, our preparations were fixed with glutaraldehyde. Hay & Dodson, (1973) reported that preparations fixed with glutaraldehyde and osmium show collagen fibres with a period of 55–60 nm rather than the 64 nm reported in non-fixed collagen fibrils, representing a shortening of 6·3 to 14 % of the length of the fibril in fixed material (Gross, 1956). Also, the tri-dimensional localization of the SLS within

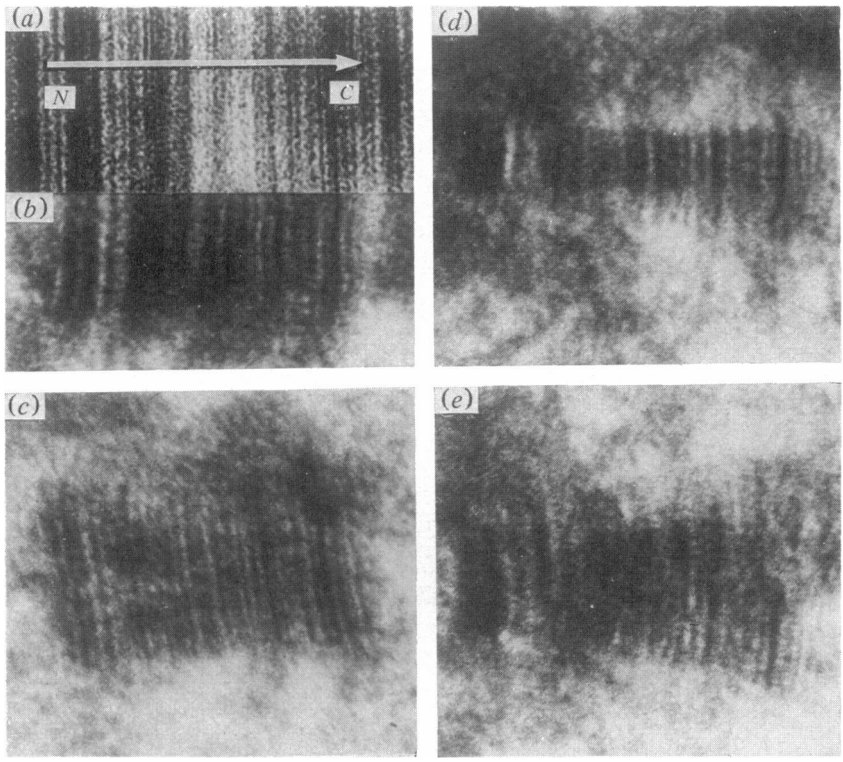

Fig. 5. (*a*) Segment of a polymeric SLS obtained from calf skin collagen. (From Hodge (1967) reproduced by permission of the author and publisher). N and C denote amino and carboxy termini. (*b*) Detail of Fig. 4 (arrow): intracellular SLS located in a cytoplasmic vacuole matched with the main bands of calf skin collagen SLS. ×170000. (*c–e*) Additional intracellular SLS crystallites embedded in electron-dense material and located in cytoplasmic vacuoles, from colchicine-treated preparations. (*c*) ×250000. (*d*) ×210000. (*e*) ×210000.

the vacuoles is an additional source of distortion. The dimensions of our intracellular SLS corrected for these factors, and the presence of the main bands at the amino and carboxy termini, are compatible with intact TC molecules. The absence of additional bands in the SLS crystallites suggests that the conversion of procollagen to collagen has occurred in the intravacuolar population of molecules (Fig. 5). The finding of intracellular SLS is intriguing, since these aggregates are usually formed *in vitro* when ATP is added to a solution of collagen at low pH (Schmitt *et al.* 1953). Preparation artifacts, with loss of integrity of the cell membrane, are not likely since the vacuoles containing SLS were found *in vitro* and *in vivo*, while evidence of cytotoxicity was not observed in our experiments. It is possible that the intracellular SLS could be the result of phagocytosis of exogenous extracellular fibres, since intracellular collagen fibres have been demonstrated by electron microscopy in many systems undergoing rapid collagen degradation (Harris & Krane, 1974; Perez-Tamayo, 1970; Woessner, 1968). Several lines of evidence, however, suggest that the intracellular SLS found in our study are not the result of endocytosis of exogenous collagen fibres. Though all cells are capable of phagocytic activity, this is usually not a prominent feature in fibroblasts (Movat & Fernando, 1962). Colchicine has been shown to inhibit particle uptake and alter aspects of the phago-

cytic process (Lehrer, 1973), though in some systems it may not interfere with phagocytosis *per se* (Malawista & Bensch, 1967). Moreover, it did not enhance phagocytosis in any of the reports on collagen secretion (Ehrlich, Ross & Bornstein, 1974; Olsen & Prockop, 1974; Scherft & Heersche, 1975). The observation of intact SLS within cytoplasmic vacuoles suggests that the population of TC forming these SLS had not yet undergone crosslinking. It has been postulated that, in states of rapid collagen resorption, fragments consisting of many molecules may be split off from the fibre by collagenases and then phagocytosed and completely degraded within phagolysosomes (Harris & Krane, 1974). This is a possibility, since colchicine has also been shown to increase collagenolytic activity (Harris & Krane, 1971). This mechanism, however, does not explain the formation of SLS within cellular vacuoles which requires the presence of intact free TC molecules. Perez-Tamayo (1972) has reported the presence of SLS in tissue undergoing rapid collagen degradation *in vivo*, extracellularly and occasionally intracellularly. In our study SLS crystallites were only found in intracellular vacuoles and none were found extracellularly. The recent observation by Imura, Tanaka & Takase (1975) of SLS in Golgi-derived vacuoles in tumour cells, and the old report by Sheldon & Kimball (1962) of FLS in vacuoles from chondrocytes may be related phenomena. What is then the significance of the massive formation of intravacuolar SLS (Figs. 4, 5) under the influence of colchicine? Our findings may be related to observations of cellular autophagocytosis reported in secretory cells (De Duve & Wattiaux, 1966; Ericsson, 1969) and may thus help to understand the fate of procollagen retained intracellularly when colchicine blocks the secretory process. In autophagic events, portions of the cytoplasm, organelles, or products of the cell, through various mechanisms, enter the lysosomal system of the secretory cell and are degraded by lysosomal enzymes. For instance, through experimental manipulation of the secretory cycle of the pancreatic acinar cell, evidence has been obtained for the participation of the lysosomal system in the processing of stored secretory material (Hand, 1972). Smith & Farquhar (1966) similarly described the complete digestion of the secretory granules in endocrine cells of the anterior pituitary gland. In addition, drugs known to interfere with microtubules have also been reported to induce autophagocytosis (Hirsimaki, Arstila & Trump, 1975). Even if the vacuoles containing SLS are secretory, it is possible that inhibition of transport by colchicine leads to the fusion of protease-containing vesicles with procollagen containing vacuoles, a situation peculiar to colchicine-treated preparations. Present views on the conversion of procollagen to collagen support the existence of more than one enzyme involved in this process (Davidson, McEneany & Bornstein, 1975; Fessler, Morris & Fessler, 1975; Tanzer *et al.* 1974) which is thought to occur extracellularly (Bornstein, 1974). Though the mechanism of extrusion of procollagen peptidases is not known it is possible that it may be mediated via small vesicles normally discharged to the extracellular space after fusing with the cell membrane or with procollagen containing vacuoles just prior to extrusion. Procollagen retained within the cell in cytoplasmic vacuoles would enter into contact with procollagen peptidases or with lysosomal proteases leading to the intravacuolar conversion of procollagen to collagen as a first step in the intracellular degradation of the secretory product blocked by the action of colchicine.

SUMMARY

Corium fragments incubated *in vitro* with colchicine or obtained from chick embryos 11 days old after *in vivo* injection of this alkaloid were studied with the electron microscope. The well known effect of colchicine in producing an accumulation of procollagen was demonstrated by electrophoretic separation of the collagenous products in polyacrylamide gels after incubation of corium fragments *in vitro* with labelled amino acids. Electron microscopical studies showed various types of cytoplasmic vacuoles containing collagenous fibrillar structures. Some of these vacuoles contained non-striated filaments, fibres with the periodicity of native collagen, and segment long spacing (SLS) crystallites, embedded in an electron-dense substance. The central aspect of this communication is the finding of intracellular SLS in cytoplasmic vacuoles. These crystallites were morphologically intact, and had no additional bands at the NH_2 or COOH ends, suggesting that they were formed by the aggregation of tropocollagen molecules. Since the presence of intact SLS in cytoplasmic vacuoles suggests that these are secretory, we believe that inhibition of the transcellular transport system by colchicine permitted the visualization of vacuoles containing the products of tropocollagen interconversions. We interpret these findings as an example of autophagocytosis, with intracellular processing of procollagen, occurring in chick embryo fibroblasts when the secretion of procollagen is inhibited by colchicine.

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