Synthesis of Procollagen by Matrix-Free Cells from Embryonic-Chick Arteries

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Cells were isolated from the major arteries of 17-day chick embryos by digestion of the tissue with collagenase and trypsin. The cells, when examined immediately after isolation, exhibited a high degree of viability and they were shown to synthesize and secrete procollagen at a high and constant rate for several hours when incubated in suspension in modified Krebs medium. Continuous labelling of the cells with [¹⁴C]proline demonstrated a lag of about 30 min between the time at which the synthesis of non-diffusible peptide-bound hydroxy[¹⁴C]proline became linear and the time at which its secretion into the medium became linear. This lag time compares with that of 18 min observed for freshly isolated matrix-free cells from embryonic-chick tendon, which synthesize and secrete the same type of collagen. Gel-filtration chromatography and polyacrylamide-gel electrophoresis indicated that the collagenous polypeptides secreted into the medium were in the precursor form, known as procollagen, and that the constituent pro- α -chains were linked by interchain disulphide bonds and were also in a triple-helical conformation. Characterization of the secreted procollagen by gel-filtration chromatography, polyacrylamide-gel electrophoresis, DEAE-agarose chromatography, and polyacrylamide-gel electrophoresis of peptides obtained by CNBr cleavage, indicated that the predominant form was type-I procollagen. This work extends the range of freshly isolated matrix-free cell systems, which have been characterized for use in studies on the biosynthesis and secretion of procollagen, and it indicates differences in the rates of secretion of procollagen in different cell types secreting the same type of procollagen.

It is now well established that there are several genetically distinct types of collagen, which are apparently tissue-specific (Miller, 1973; Miller & Matukas, 1974; Grant *et al.*, 1975; Epstein & Munderloh, 1975). Each of the different types of collagen is initially synthesized by connective-tissue cells as a precursor form known as procollagen (for reviews see Schofield & Prockop, 1973; Bornstein, 1974; Grant *et al.*, 1975; Martin *et al.*, 1975). Much information about the various steps in the intracellular biosynthesis, transport and secretion of the different types of procollagen has come from the use of matrix-free single-cell suspensions isolated from various embryonic-chick tissues by digestion with collagenase and trypsin (see Grant *et al.*, 1975), a technique originally developed by Dehm & Prockop (1971). Thus type-I procollagen synthesis and secretion has been studied by using freshly isolated cells from leg tendons (Dehm & Prockop, 1971), that of type-II procollagen by using

cells from sternal cartilage (Dehm & Prockop, 1973) and that of type-IV procollagen by using cells from the eye lens (Grant *et al.*, 1972*a,b*). Such cell systems offer certain advantages over other collagen-synthesizing systems in that the suspended cells remain viable and synthesize protein at rates comparable with those of cells *in situ* within tissues for several hours, with a significant proportion of the protein synthesized being procollagen. Because of the removal of the extracellular matrix, which would otherwise interfere, they are amenable to disruption and subsequent isolation of subcellular organelles, thus permitting studies of procollagen biosynthesis at a subcellular level, and they can be used to study intracellular and extracellular events separately (Grant *et al.*, 1975).

In the present study we have examined the synthesis and excretion of procollagen by cells freshly isolated from the major arteries of the chick embryo, thus extending the number of cell types available as experimental systems for examining the mechanisms of procollagen synthesis and secretion. It was originally thought that the artery cells might provide a system for studying the synthesis of type-III procollagen,

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since it had previously been shown that human aorta contains significant amounts of this collagen (Chung & Miller, 1974; Trelstad, 1974). However, our experiments designed to characterize the procollagen secreted by the artery cells indicate that they synthesize predominantly type-I procollagen. The secretion of type-I procollagen by these artery cells, however, appears to be considerably slower than the secretion of type-I procollagen by similarly prepared cells from embryonic-chick tendon.

A preliminary account of part of this work has been presented (Schofield & Harwood, 1975).

Materials and Methods

Materials

Fertile eggs were purchased from Mytholmroyd Hatcheries, Hebden Bridge, Yorks., U.K., and incubated in a moist atmosphere at 37°C for 17 days in a Westernette egg incubator. L-[U-¹⁴C]Proline (290 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin solution (2.5%, w/v) in 0.15M-NaCl, Eagle's basal medium with Earle's salts and bovine foetal calf serum were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Crude bacterial collagenase (type II), pepsin (twice-crystallized), phenylmethanesulphonyl fluoride, cycloheximide and *N*-ethylmaleimide were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and acrylamide, bisacrylamide and CNBr were from Eastman Organic Chemicals, Liverpool, U.K. Agarose beads (6%; Bio-Gel A-5m, 200–400 mesh) and DEAE-agarose were from Bio-Rad Laboratories, Bromley, Kent, U.K., and activated thiol-Sepharose 4B was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Sodium dodecyl sulphate, which was specially purified electrophoresis grade, and all other reagents, which were of analytical reagent grade when possible, were obtained from BDH, Poole, Dorset, U.K.

Type-I and type-III collagens were extracted from embryonic-chick skin by limited digestion with pepsin and they were separated by covalent chromatography on activated-thiol-Sepharose 4B (Sykes, 1976). The skin was extracted with 2.5M-NaCl for 24h at 4°C before pepsin treatment to remove soluble non-collagenous proteins. [¹⁴C]Proline-labelled procollagen secreted into the medium by confluent cultures of human skin fibroblasts was given by Dr. C. H. J. Sear, Department of Medical Biochemistry, University of Manchester, and it was prepared as described by Sear *et al.* (1976). The human skin fibroblast line was the F7000 line supplied by Flow Laboratories.

Isolation of matrix-free cells from embryonic-chick arteries

The major arteries from 17-day embryos were dissected as previously described (Murphy *et al.*, 1972), and freshly isolated cells were prepared from this tissue by a method similar to that described by Dehm & Prockop (1971). In a typical experiment arteries from approx. 100–120 embryos were digested by incubation with 50mg of crude bacterial collagenase and 1.0ml of trypsin solution, which were added to 10ml of Eagle's basal medium under an atmosphere of O₂/CO₂ (19:1) for 60–90 min at 37°C with constant shaking. The matrix-free cells were harvested and washed as described previously (Dehm & Prockop, 1972).

Incubation of matrix-free cells

The washed cells were resuspended in modified Krebs medium II either with or without 10% foetal calf serum as indicated below. A small sample was taken for cell counting in a haemocytometer, and the cell density was adjusted to 1 × 10⁷ cells/ml. Incubations were carried out under air at 37°C, with moderate shaking, in either silicone-treated glass or Teflon Erlenmeyer flasks. The cells were pre-incubated for 15–30 min before addition of [¹⁴C]-proline and the incubations were then continued for the times indicated.

In experiments in which the rates of radioisotope incorporation into polypeptides in the cells and medium were measured, the medium contained 10% (v/v) foetal calf serum, and incubations were stopped by adding portions of the cell suspension to 0.1 vol. of a solution containing cycloheximide and 2,2'-bipyridine in modified Krebs medium II to give final concentrations of 100 µg/ml and 1 mM respectively (Dehm & Prockop, 1971). The cells were separated from the medium by centrifugation at 1200g for 10 min at room temperature (20°C). In experiments to prepare secreted polypeptides, foetal calf serum was omitted from the medium and, after incubation of the cells with [¹⁴C]proline for 4–6 h, the incubation was terminated by separating the cells from the medium by centrifugation at 1200g for 10 min at room temperature without addition of inhibitors.

Preparation of secreted procollagen polypeptides

Secreted procollagen polypeptides were prepared for chromatographic and electrophoretic examination, and for pepsin treatment, by precipitating them from the media with (NH₄)₂SO₄. Immediately after separation from the cells the media were treated with phenylmethanesulphonyl fluoride, *N*-ethylmaleimide and EDTA, to final concentrations of 2 mM, 10 mM and 25 mM respectively, to inhibit proteolysis (Monson & Bornstein, 1973). The media were dialysed against 0.4M-NaCl/0.1M-Tris/HCl, pH 7.4

at 4°C, for 24h, and procollagen was precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation (Schofield *et al.*, 1974).

Gel-filtration chromatography in sodium dodecyl sulphate

Gel-filtration chromatography was carried out under denaturing conditions on a column (15mm×900mm) of 6% agarose beads in the presence of sodium dodecyl sulphate as described previously (Jimenez *et al.*, 1971; Schofield *et al.*, 1974). Samples were prepared for chromatography by treating them with 1% (w/v) sodium dodecyl sulphate in 0.01M-sodium phosphate buffer, pH7.4, in the presence or absence of 1% (v/v) 2-mercaptoethanol at 100°C for 3 min (Schofield *et al.*, 1974).

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

Disc gel electrophoresis of secreted procollagen polypeptides and pepsin-treated procollagen was performed in a Shandon Southern Instruments (U.K.) apparatus by using 5% (w/v) polyacrylamide gels (6mm×60mm) in the presence of sodium dodecyl sulphate by the method of Weber *et al.* (1972). Electrophoresis was carried out at a current of 6mA per tube for 4–5h. Disc gel electrophoresis of CNBr peptides was carried out in a similar way except that 10% polyacrylamide gels (6mm×90mm) were used. The band patterns of Coomassie Brilliant Blue-stained gels were recorded by using a Joyce–Loebl Chromoscan densitometer. Gels were sliced for localization of radioactivity with a Mickle gel slicer, which was set to cut 1 mm slices.

Limited pepsin cleavage

$(\text{NH}_4)_2\text{SO}_4$ -precipitated procollagen from the medium (see above) was suspended in 0.5M-acetic acid containing pepsin (100 µg/ml) at 4°C. The sample was stirred at 15°C for 6h and dialysed against 0.4M-NaCl/0.1M-Tris/HCl, pH7.4, for a further 24h at 4°C to inactivate the pepsin. Samples for gel-filtration chromatography were treated directly with sodium dodecyl sulphate, whereas samples for disc gel electrophoresis were first concentrated by precipitation of the pepsin-treated procollagen by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation before treatment with sodium dodecyl sulphate. Samples of pepsin-treated procollagen, which were to be subsequently cleaved by CNBr, were dialysed against 1M-CaCl₂/0.05M-Tris/HCl, pH7.5, after inactivation of the pepsin by dialysis against the NaCl/Tris buffer.

DEAE-agarose chromatography

$(\text{NH}_4)_2\text{SO}_4$ -precipitated procollagen samples were dissolved by stirring at 4°C in 0.01M-Tris/HCl (pH7.4)/4M-urea and they were dialysed against the

same buffer. The samples were then applied to a column (10mm×150mm) of DEAE-agarose at 18°C. The column was eluted for a short time with the sample buffer, and the [¹⁴C]proline-labelled procollagens were eluted with a linear salt gradient (0–0.3M-NaCl) over a total volume of 100ml (Lichtenstein *et al.*, 1975).

CNBr cleavage

Pepsin-treated procollagen polypeptides were chromatographed on a 6% agarose column (15mm×900mm) eluted with 1M-CaCl₂/0.05M-Tris/HCl, pH7.5 (Piez, 1968) in order to purify the collagen α-chains. The sample was reduced with 2-mercaptoethanol (2%, v/v, final concentration) and denatured by heating at 100°C for 5 min before application to the column. The material eluted in the region corresponding to α-chains was pooled, dialysed against 1% (v/v) acetic acid and freeze-dried. CNBr cleavage was carried out as described by Byers *et al.* (1974). CNBr was removed by freeze-drying.

Assay procedures

Radioactivity in column-chromatographic fractions was determined by adding 0.1ml samples to 10ml of the solvent system described by Juva & Prockop (1966) and counting in a liquid-scintillation counter at an efficiency of about 90%. Radioactivity in polyacrylamide-gel slices was determined in a similar way after dissolution of the slices in 0.4ml of 30% (v/v) H₂O₂ at 60°C for 18h. Hydroxy[¹⁴C]-proline radioactivity in fractions of medium and cells or in chromatographic fractions was determined by a specific radiochemical procedure (Juva & Prockop, 1966) after hydrolysis of the samples in 6M-HCl for 18h at 105°C in sealed Pyrex tubes. In experiments to determine the kinetics of synthesis of [¹⁴C]proline-labelled polypeptides, of non-diffusible peptide-bound hydroxy [¹⁴C]proline and of secretion of peptide-bound hydroxy[¹⁴C]proline, the separated fractions of medium and cells were dialysed against running tap water for 24h to remove unincorporated [¹⁴C]proline, and the non-diffusible materials were hydrolysed as above.

Experimental and Results

Preparation of freshly isolated matrix-free cells from chick arteries

Preliminary experiments indicated that cells, over 90% of which were viable as judged by the Trypan Blue-exclusion test (Dehm & Prockop, 1971), could be isolated from embryonic-chick arteries by digestion with crude collagenase and trypsin. They were similar in size to cells prepared in a similar manner from embryonic-chick sternal cartilage (Dehm & Prockop, 1973), but were smaller than those from embryonic-chick tendon (Dehm & Prockop, 1971). The cell size

was somewhat variable and occasional cells had cytoplasmic blebs. Although, as initially isolated, most of the cells were present in suspension as single cells, they had a marked tendency to aggregate. However, this tendency was lessened by addition of 10% (v/v) foetal calf serum to the medium in which they were suspended. Yields of cells were similar to those for embryonic-chick sternal cartilage, and in most experiments between 5×10^6 and 7×10^6 cells were obtained per embryo. Experiments indicated that neither the cell yields nor those properties of the cells described above were altered by the use of purified collagenase in the digestion of the tissues, used previously for preparation of freshly isolated cells from chick tissues (Dehm & Prockop, 1971; 1973; Grant *et al.*, 1972a,b).

Time-course for synthesis and secretion of total peptide-bound ^{14}C and peptide-bound hydroxy[^{14}C]-proline

To investigate the kinetics of synthesis and secretion of collagen, freshly isolated matrix-free cells were labelled continuously with [^{14}C]proline. Samples were withdrawn at 20 min intervals and pipetted directly into cycloheximide and 2,2'-bipyridine to stop protein synthesis and to stop hydroxylation of proline (Dehm & Prockop, 1971). The cells were then separated from the medium, and, after dialysis to remove unincorporated [^{14}C]proline, the total non-diffusible polypeptide-bound ^{14}C and non-diffusible polypeptide-bound hydroxy[^{14}C]proline radioactivity were determined in both cells and medium.

The results of such experiments indicated that the synthesis of ^{14}C -labelled polypeptides and the synthesis of non-diffusible peptide-bound hydroxy[^{14}C]proline were linear with time for several hours (Fig. 1). In two separate experiments lag periods of about 7 and 9 min were observed between the time at which incorporation of ^{14}C into polypeptides (cells plus medium) became linear and the time at which the accumulation of total peptide-bound hydroxy[^{14}C]proline (cells plus medium) became linear (i.e. abscissa-intercept values of least-squares regression lines; Fig. 1). There was also a considerable lag (about 29 and 30 min in two separate experiments) between the time at which the accumulation of peptide-bound hydroxy[^{14}C]proline in the medium became linear. In the experiments shown in Fig. 1, hydroxy[^{14}C]proline accounted for about 20% of the total ^{14}C in the cells and it accounted for about 39% of the total ^{14}C in the medium. In the total system (cells plus medium) about 27% of the total ^{14}C was accounted for as hydroxy[^{14}C]proline.

Size and triple-helical nature of the secreted collagen polypeptides

The size of the collagen polypeptides secreted

Fig. 1. Time-course of incorporation of [^{14}C]proline and synthesis and secretion of non-diffusible hydroxy[^{14}C]proline by cells isolated from the arteries of 17-day chick embryos

Artery cells (8×10^7) were incubated in 16 ml of modified Krebs medium II plus 10% (w/v) foetal calf serum with $16 \mu\text{Ci}$ of [^{14}C]proline, and samples (2.5 ml) were withdrawn at the times indicated. The linear regression lines were calculated by the method of least squares and they were extrapolated (broken lines) to the abscissa. The arrows indicate the abscissa-intercept values of the linear regression lines. (a) Total non-diffusible ^{14}C ; (b) non-diffusible hydroxy[^{14}C]proline. ●, Cells plus medium; ▲, cells; ■, medium.

into the medium by the artery cells was investigated by chromatographing them on 6% agarose in the presence of sodium dodecyl sulphate. When the [^{14}C]proline-labelled polypeptides from the medium were chromatographed on 6% agarose before reduction, a substantial proportion of the ^{14}C was eluted in the void volume of the column, indicating that it had a mol.wt. in excess of 300 000 (Fig. 2a). When the same material was reduced with 2-mercaptoethanol before chromatography, the void-volume peak was abolished and a new peak was eluted in the same position as pro- α -chains from embryonic-chick tendon cells (Fig. 2b). This peak contained essentially

Fig. 2. Gel-filtration chromatography of [^{14}C]proline-labelled polypeptides secreted by freshly isolated matrix-free artery cells

Artery cells (7.2×10^8) were incubated in 70 ml of modified Krebs medium II with $10 \mu\text{Ci}$ of [^{14}C]proline for 6 h. The polypeptides from the medium were precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation (176 mg/ml) after division of the medium into three equal parts. (a) Sample chromatographed without prior reduction; (b) sample chromatographed after reduction with 2-mercaptoethanol; (c) sample chromatographed after pepsin treatment but without prior reduction. ●, Total ^{14}C in each fraction; ○, hydroxy[^{14}C]proline in each fraction. The arrows indicate: 1, the void volume (V_0) of the column; 2, the elution position of β -chains of collagen; 3, the elution position of pro- α -chains of procollagen secreted by embryonic-chick tendon cells; 4, the elution position of α -chains of collagen.

all of the hydroxy[^{14}C]proline previously eluted in the void volume. The findings with regard to the elution positions of the unreduced and reduced poly-

peptides, together with the fact that hydroxy[^{14}C]proline accounted for about 50% of the total ^{14}C in these peaks, indicates that the void-volume peak consisted of procollagen pro- α -chains linked together by interchain disulphide bonds.

In a parallel experiment another portion of the polypeptides from the medium was subjected to treatment with pepsin at 15°C , conditions under which the triple-helical structure of native collagen is resistant to digestion. When this material was chromatographed on the 6% agarose column without reduction, a new peak was observed which chromatographed in the position of collagen α -chains (Fig. 2c). This peak again contained essentially all the hydroxy[^{14}C]proline previously eluted at either the void volume or the position of pro- α -chains. It is noteworthy that little or no hydroxy[^{14}C]proline-containing material was eluted in a position corresponding to a molecular weight higher than that of β -chains of collagen, even though the sample was not reduced before chromatography (Fig. 2c). These observations indicated that the hydroxy[^{14}C]proline-containing pro- α -chains were secreted in a triple-helical conformation, and that no disulphide-linked α -chains were present after pepsin treatment.

Polyacrylamide-gel electrophoresis of the secreted collagen peptides

The [^{14}C]proline-labelled collagen polypeptides excreted into the medium were examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Two major bands, which corresponded to pro- α 1- and pro- α 2-chains of procollagen, were observed in either gels stained with Coomassie Brilliant Blue or gels sliced for radioactive counting. Measurement of the area beneath the densitometer peaks corresponding to these bands gave a pro- α 1/pro- α 2 ratio of 1.7:1. The ratio of radioactivity in the pro- α 1-band to that in the pro- α 2-band was 2.1:1 (Fig. 3).

Similar experiments were also carried out after treatment of the procollagen polypeptides from the medium with pepsin under conditions in which the triple-helical portion of the molecule is resistant to digestion, and which result in the conversion of pro- α -chains into polypeptides of approximately the same size as those of α -chains of collagen. When the pepsin-treated samples were examined two new bands corresponding to α 1- and α 2-chains of collagen were observed in both stained gels and gels sliced for radioactive counting (Figs. 3a and 3b). The ratio of α 1- to α 2-chains, as determined by measurement of the area beneath the densitometer peaks and also by determination of the radioactivity in each peak, was again 2.1:1. Similar values were obtained for two other preparations of [^{14}C]proline-labelled procollagen from the medium of the artery

Fig. 3. Polyacrylamide-gel electrophoresis of [^{14}C]proline-labelled polypeptides secreted by freshly isolated matrix-free artery cells

Artery cells (5×10^8) were incubated in 50 ml of modified Krebs medium II with $10 \mu\text{Ci}$ of [^{14}C]proline for 4 h. The ^{14}C -labelled polypeptides in the medium were precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ (176 mg/ml) and electrophoresed on 5% polyacrylamide gels. (a) Densitometer tracings of Coomassie Brilliant Blue-stained gels. —, Sample electrophoresed before pepsin treatment but after reduction with 2-mercaptoethanol; ----, sample electrophoresed after treatment with pepsin. (b) Distribution of ^{14}C in 1 mm gel slices. ●, Sample electrophoresed before pepsin treatment but after reduction; ○, sample electrophoresed after pepsin treatment.

cells and also in similar experiments with procollagen secreted by embryonic-chick tendon cells.

Chromatography of secreted procollagens from matrix-free artery cells on DEAE-agarose

Procollagen secreted into the medium by matrix-free artery cells and procollagen secreted by human skin fibroblast cultures were chromatographed on DEAE-agarose by a method similar to that described by Lichtenstein *et al.* (1975). The procollagen from human skin fibroblasts was resolved into two peaks (Fig. 4a) which have been shown to be type-I procollagen (the major peak) and type-III procollagen (the minor peak) (Lichtenstein *et al.*, 1975). By contrast, procollagen secreted by the artery cells was

eluted in one peak only, in the same position as type-I procollagen from skin fibroblast medium (Fig. 4b). It appeared from this experiment therefore that no significant amount of type-III procollagen was present in the medium from the matrix-free artery cells.

Polyacrylamide-gel electrophoresis of peptides derived by CNBr cleavage of pepsin-treated procollagen polypeptides from the medium

Secreted procollagen polypeptides were further characterized by polyacrylamide-gel electrophoresis of the peptides derived from them by CNBr cleavage. The secreted procollagen polypeptides were treated with pepsin at 4°C to remove the N- and C-terminal

Fig. 4. DEAE-agarose chromatography of procollagen secreted into the medium by matrix-free artery cells and cultured human skin fibroblasts

Artery cells (5.3×10^8) were incubated in modified Krebs medium II plus 10% foetal calf serum (50ml) with $10 \mu\text{Ci}$ of [^{14}C]proline for 2h. Confluent cultures of F7000 human skin fibroblasts were incubated with $5 \mu\text{Ci}$ of [^{14}C]proline for 24h. Procollagen was precipitated from the media with $(\text{NH}_4)_2\text{SO}_4$ and chromatographed on DEAE-agarose as described in the text. (a) Procollagen from the medium of F7000 human skin fibroblasts; (b) procollagen secreted into the medium by matrix-free artery cells. ----, NaCl gradient used to elute the procollagen.

Fig. 5. Polyacrylamide-gel electrophoresis of peptides derived by CNBr cleavage of type-I collagen, type-III collagen and pepsin-treated procollagen secreted by matrix-free artery cells

Artery cells (6.5×10^8) were incubated in 65ml of modified Krebs medium II with $10 \mu\text{Ci}$ of [^{14}C]proline for 4h. The ^{14}C -labelled polypeptides in the medium were concentrated and treated with pepsin, and the resultant ^{14}C -labelled α -chains were purified and cleaved with CNBr as described in the text. CNBr peptides from type-I and type-III collagens of embryonic-chick skin were electrophoresed in parallel gels for comparison. (a) Distribution of radioactivity in [^{14}C]proline-labelled CNBr peptides from pepsin-treated procollagen secreted by matrix-free artery cells; (b) densitometer tracing of CNBr peptides from embryonic-chick skin type-I collagen; (c) Densitometer tracing of CNBr peptides from embryonic-chick skin type-III collagen.

extension peptides, and the derived α -chains were purified by gel-filtration chromatography before CNBr cleavage. CNBr peptides from type-I and type-III collagen of embryonic-chick skin were electrophoresed in parallel gels for comparison with those from the collagen secreted by the artery cells.

The distribution of radioactivity in gels in which the CNBr peptides from the pepsin-treated procollagen secreted by the chick artery cells were separated is illustrated in Fig. 5(a). Densitometer tracings of the gels in which the CNBr peptides from type-I and type-III collagens were electrophoresed are presented in Figs. 5(b) and 5(c). The CNBr peptide patterns of

type-I and type-III collagen were quite different and characteristic for the type of collagen (Figs. 5b and 5c). Comparison of these patterns with that for the chick artery collagen (Fig. 5a) indicated an obvious similarity between the patterns for type-I collagen from embryonic-chick skin and that for collagen from the artery cells. There was little similarity between the patterns for peptides derived from the collagen secreted by the artery cells and the pattern for those from embryonic-chick skin type-III collagen.

Discussion

Freshly isolated matrix-free cell systems offer significant advantages over other systems for investigation of certain aspects of collagen biosynthesis (Grant *et al.*, 1975). In the present study we have carried out experiments to develop such a cell system from embryonic-chick arteries for use in studying the synthesis of extracellular-matrix components in vascular tissue, and a characterization of the collagen polypeptides secreted by the artery cells has been carried out.

Digestion of the major arteries from 17-day chick embryos with trypsin and crude collagenase resulted in the release of single cells which remained viable and which synthesized and secreted collagen at a rapid rate for several hours. About 27% of the total peptide-bound ^{14}C in the cells plus medium was in hydroxy- ^{14}C proline after continuous labelling of the cells with ^{14}C proline. If all of this hydroxy- ^{14}C proline had been in collagen polypeptides this would have indicated that approx. 50–55% of the ^{14}C proline incorporated by these cells was in collagen polypeptides, which would be similar to the value for freshly isolated embryonic-chick cartilage cells (Dehm & Prockop, 1973). However, in a report (Uitto *et al.*, 1976) that appeared at the completion of this work evidence was presented indicating that a protein with the properties of tropoelastin was secreted by cells prepared from embryonic-chick arteries by techniques similar to those used here. This elastin component contained a significant amount of hydroxyproline in that 33% of the total imino acid content of this component was hydroxyproline. This value is considerably higher than that of about 13% obtained in another study for tropoelastin secreted by embryonic-chick artery cells prepared by similar techniques (Rosenbloom & Cywinski, 1976), and higher than the values obtained by chemical analysis of elastins from a variety of tissues, and in particular from chick arterial tissue (Sandberg *et al.*, 1969; Smith *et al.*, 1972; Sykes & Partridge, 1974; Foster *et al.*, 1975). In our own experiments we have also observed that peptide-bound hydroxy- ^{14}C proline is present in the medium in a peak, the elution position of which indicates considerably lower

molecular weight than that of collagenous polypeptides (Fig. 2), but we have not characterized this material further. Nevertheless, in the experiment reported here in which the rates of synthesis and secretion of peptide-bound hydroxy- ^{14}C proline were examined (Fig. 1) there was little evidence of components with different rates of synthesis and secretion, which would have been apparent as an inflexion in the plots of the data for peptide-bound hydroxy- ^{14}C proline in the cells plus medium and of the data for peptide-bound hydroxy- ^{14}C proline in the medium alone. This presumably means that both the collagen component and the elastin component are hydroxylated and secreted at similar rates. The experiments carried out by Uitto *et al.* (1976), however, show that inhibition of procollagen synthesis by incubation of artery cells with proline analogues does not affect the secretion of the elastin component, indicating that procollagen secretion and elastin secretion are probably not interdependent.

The lag times observed for the artery cells between the time at which total hydroxy- ^{14}C proline synthesis became linear and the time at which the accumulation of hydroxy- ^{14}C proline in the medium became linear (29 and 30 min in two experiments; see Fig. 1) are considerably longer than that of 18 min observed for freshly isolated embryonic-chick tendon cells (Dehm & Prockop, 1972). The lag times are similar to that of 33 min observed in freshly isolated cells from embryonic-chick cartilage (Dehm & Prockop, 1973) and much shorter than that of about 60 min observed in cells from embryonic-chick lens (Grant *et al.*, 1972a). Rosenbloom & Cywinski (1976), however, have reported a lag time of only 23 min for freshly isolated cells from embryonic-chick arteries, and this value is closer to that found in embryonic-chick tendon cells (Dehm & Prockop, 1971). The reasons for this discrepancy between their results and those presented here are not known. The long lag time observed here is a somewhat surprising finding, since the artery cells appear to be synthesizing type-I collagen (see below) as do tendon cells, whereas cartilage cells secrete type-II collagen (Dehm & Prockop, 1973).

It had previously been suspected that the lag between synthesis and secretion of different types of collagen might be related to the different extents of hydroxylation of lysine and glycosylation of hydroxylysine, which are apparent in different collagen types (Grant *et al.*, 1972a, 1975; Dehm & Prockop, 1973), although the evidence to support this suggestion is rather indirect. However, the observation that there are different secretion times in two different cell types both synthesizing the same type of collagen throws some doubt on this interpretation and suggests that other factors may be involved in determining how long the lag between synthesis and

secretion will be. Exactly what these factors are remains to be seen. Evidence obtained about the relationship between interchain disulphide-bond formation and the formation of the triple-helical structure in procollagen and the demonstration that these events occur at different times during procollagen biosynthesis in different cell types strongly suggests that the timing of these events, or the subcellular site at which they occur, may be of considerable importance in determining the time taken to secrete the completed procollagen molecule in different cell types (Grant *et al.*, 1975).

The collagenous polypeptides secreted by the artery cells were shown to be in the form of procollagen by gel-filtration chromatography and polyacrylamide-gel electrophoresis of reduced polypeptides from the medium (Figs. 2b and 3). Gel-filtration chromatography of unreduced polypeptides from the medium (Fig. 2a) illustrated that essentially all the procollagen recovered from the medium was in a form in which the pro- α -chains were linked together by interchain disulphide bonds. That the procollagen polypeptides were in a triple-helical conformation was also demonstrated by the fact that, after treatment of the secreted procollagen with pepsin under conditions in which the triple-helical portion of the molecule is resistant to digestion, essentially all of the procollagen polypeptides were now recovered as material the size of α -chains of collagen by both gel-filtration chromatography (Fig. 2c) and polyacrylamide-gel electrophoresis (Fig. 3). These findings are similar to those for procollagen secreted into the medium by freshly isolated cells from embryonic-chick lens (Grant *et al.*, 1973), and tendon cells (Schofield *et al.*, 1974) in which the pro- α -chains are linked by disulphide bonds and which are also present in the triple-helical conformation.

Further characterization of the procollagen secreted into the medium indicated that it was predominantly type-I procollagen. Gel-filtration chromatography of pepsin-treated procollagen from the medium without prior reduction gave no evidence of any significant amount of material eluted at a position corresponding to mol.wt. 300000 (Fig. 2c). Type-III collagen has been shown to be present in other tissues as a high-molecular-weight aggregate in which the three α -chains comprising the molecule are linked by interchain disulphide bonds and in which the disulphide bonds are located in a pepsin-resistant region (Chung & Miller, 1974). Therefore one must conclude from this experiment that either type-III procollagen is not synthesized by the artery cells or if type-III procollagen is present, it does not contain disulphide bonds after pepsin treatment.

Polyacrylamide-gel electrophoresis of reduced procollagen polypeptides from the medium showed that two major bands were present corresponding to pro- α 1- and pro- α 2-chains and that the ratio of

pro- α 1- to pro- α 2-chain was close to 2, estimated by using either the area beneath the peaks on densitometer tracings or the radioactivity in gel slices (Fig. 3). Similarly, when polyacrylamide-gel electrophoresis of pepsin-treated procollagen from the medium was carried out, two major components were again observed, this time corresponding to α 1- and α 2-chains of collagen (Fig. 3). The ratio of α 1- to α 2-chains was again close to 2. If type-III collagen had been present in significant amounts the ratio would have been significantly greater than 2, since type-III collagen contains only one type of α -chain with properties similar to those of α 1-chains of type-I collagen (Chung & Miller, 1974; Trelstad, 1974). From these data it again appeared that only type-I procollagen was being secreted by the artery cells in significant amounts.

Chromatography of the procollagen from the medium on DEAE-agarose resulted in only one peak being observed, corresponding to the elution position of type-I procollagen (Fig. 4). In contrast, the procollagen secreted into the medium by confluent cultures of human skin fibroblasts, which synthesize both type-I and type-III collagen, was resolved into two peaks corresponding to type-I procollagen and type-III procollagen (Fig. 4). This experiment again demonstrates that the preponderant type of procollagen secreted by the artery cells is type-I procollagen.

Finally, the procollagen secreted by the artery cells was characterized by polyacrylamide-gel electrophoresis of the peptides derived from it by CNBr cleavage. The results demonstrated a close similarity between CNBr peptides from type-I collagen of embryonic-chick skin and those from pepsin-treated procollagen from the artery cells (Fig. 5). The CNBr peptide pattern of type-III collagen from embryonic-chick skin was quite distinct from that for the type-I collagen and there was no similarity between the pattern for the pepsin-treated procollagen from the artery cells and the pattern for the type-III collagen. However, these particular results do not exclude the possibility that a small proportion of the procollagen secreted by the artery cells could be of a type other than type I.

On the basis of these four different types of evidence, we conclude that the predominant type of procollagen being synthesized and secreted by the freshly isolated embryonic-chick artery cells is type-I collagen. Uitto *et al.* (1976) have also come to the same conclusion, although their characterization of the secreted molecule was rather less extensive than that carried out here. It remains a possibility that a small percentage of the procollagen secreted by these cells may be of a type or types other than type I, and it would perhaps be surprising if this were not so, in view of the findings of significant amounts of type-III collagen and type-IV collagen in pepsin-

solubilized collagen from human aorta (Chung & Miller, 1974; Trelstad, 1974).

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