The Synthesis and Secretion of Cartilage Procollagen

By RICHARD HARWOOD, ASHOK K. BHALLA, MICHAEL E. GRANT
and DAVID S. JACKSON

*Department of Medical Biochemistry, The Medical School, University of Manchester,
Oxford Road, Manchester M13 9PT, U.K.*

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1. Isolation of free and membrane-bound ribosomes from embryonic chick sternal-cartilage cells labelled for 4 min with [^{14}C]proline and their subsequent analysis for hydroxy[^{14}C]proline indicated that cartilage procollagen biosynthesis occurs on bound ribosomes. 2. Nascent procollagen polypeptides on bound ribosomes isolated from cells labelled with [^{14}C]lysine were found to contain hydroxy[^{14}C]lysine indicating that hydroxylation of lysine commences while the growing chains are still attached to the ribosomes. 3. Analysis of bound ribosomes labelled with either [^{14}C]proline or [^{14}C]lysine on sucrose density gradients indicated that cartilage procollagen is synthesized on large polyribosomes in the range 250–400S. 4. Microsomal preparations isolated from cells pulse-labelled for 4 min with [^{14}C]proline were used to determine the direction of release of nascent procollagen polypeptides. Puromycin induced the vectorial release of nascent procollagen polypeptides into the microsomal vesicles suggesting that the first step in the secretion of procollagen polypeptides is their transfer from the ribosomes through the membrane of the endoplasmic reticulum into the cisternal space. 5. The procollagen polypeptides secreted by cartilage cells were shown to be linked by inter-chain disulphide bonds. 6. Examination of the state of aggregation of pro- α chains in subcellular fractions isolated from cartilage cells labelled with [^{14}C]proline for various periods of time have provided data on the timing and location of inter-chain disulphide-bond formation. This process commences in the rough endoplasmic reticulum after the release of completed pro- α chains from membrane-bound ribosomes. Pro- α chains isolated from fractions of smooth endoplasmic reticulum were virtually all present as disulphide-bonded aggregates, suggesting that either disulphide bonding is completed in this cellular compartment, or that procollagen needs to be in a disulphide-bonded form to be transferred to this region of the endoplasmic reticulum. 7. Comparison of these results with previously published data on disulphide bonding in tendon cells suggests that the rate of inter-chain disulphide-bond formation is significantly slower in cartilage cells.

Recent evidence has indicated the existence of several genetically distinct types of collagen [for a review, see Miller & Matukas (1974)] the best characterized of which are the Type I collagens found in dermis, bone, tendon, etc. and Type II collagens characterized from several cartilagenous structures. The Type II collagens are distinguished by their chain composition [$\alpha 1(\text{II})$]₂ which contrasts with that of the more common Type I collagens which have $\alpha 1(\text{I})$ and $\alpha 2$ chains in the ratio 2:1. In addition, cartilage collagen α chains contain greater amounts of hydroxylated lysine and glycosylated hydroxylysine than are found in $\alpha 1(\text{I})$ and $\alpha 2$ chains (Miller, 1971; Strawich & Nimni, 1971).

It is now well-established that both these types of collagen are initially synthesized in a precursor form, termed procollagen, in which the constituent α chains have amino-terminal extensions which influence the chemical and physical properties of the procollagen molecule [for reviews, see Schofield &

Prockop (1973) and Bornstein (1974)]. By using matrix-free tendon cells it has been possible to study the synthesis of the constituent chains of Type I tendon procollagen, their assembly into the triple-helical molecule and subsequent secretion (Dehm & Prockop, 1971, 1972; Dehm *et al.*, 1972; Jimenez *et al.*, 1971, 1973, 1974; Harwood *et al.*, 1973, 1974a,b; Uitto & Prockop, 1973, 1974a,b; Schofield *et al.*, 1974; Olsen & Prockop, 1974). The absence of any extracellular matrix has permitted the successful application of subcellular fractionation techniques to these cells (Harwood *et al.*, 1974d) and by using such procedures evidence has been obtained to suggest that the synthesis and secretion of tendon procollagen is similar to that elucidated for other extracellular proteins [for a review, see Grant *et al.* (1974)].

In the present paper we report studies on the synthesis and secretion of cartilage procollagen and demonstrate that the association of the

cartilage pro- α chains through disulphide bonds occurs in the rough endoplasmic reticulum and is probably slower than that reported for tendon pro- α chains (Harwood *et al.*, 1973; Schofield *et al.*, 1974).

Experimental

Materials

Fertile eggs obtained from Mytholmroyd Hatcheries, Hebden Bridge, Yorks., U.K. were incubated for 17 days at 37°C in a moist atmosphere in a Westernette Egg Incubator (Western Incubators Ltd., Chelmsford, Essex, U.K.). [^{14}C]Proline (290 mCi/mmol) and [^{14}C]lysine (318 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin solution (2.5%, w/v) in 0.15M-NaCl, Eagles' Basal Medium in Earles' salts, and foetal calf serum were purchased from Flow Laboratories, Irvine, Ayrshire, U.K. Partially purified bacterial collagenase was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Cycloheximide and puromycin were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Triton X-100 (scintillation grade) was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and α, α' -bipyridyl was obtained from Calbiochem, Los Angeles, Calif., U.S.A. All other chemicals were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. and were of AnalaR grade whenever available.

Isolation and incubation of cartilage cells

Cells were isolated from the sterna of 17-day-old chick embryos by digestion with trypsin and collagenase under conditions similar to those described by Dehm & Prockop (1973). In most experiments approximately 4×10^6 – 6×10^6 cells were obtained per sternum. In short-term pulse-labelling experiments cells were incubated at 37°C at a concentration of approx. 4×10^7 – 7×10^7 cells/ml of modified Krebs medium (Dehm & Prockop, 1971), but when cells were incubated for 2h the concentration was 1×10^7 cells/ml of the medium. The incubations were conducted in silicon-treated glass Erlenmeyer flasks and cells were preincubated at 37°C for 15min before the addition of the appropriate ^{14}C -labelled amino acid. Termination of the incubations was achieved by rapid cooling to 4°C and by the addition of cycloheximide to a final concentration of 100 $\mu\text{g}/\text{ml}$.

Separation of free and membrane-bound cytoplasmic ribosomes and analysis of collagen-synthesizing polyribosomes

Cells labelled for 4min with either [^{14}C]proline or [^{14}C]lysine were collected by centrifugation at 1200g

for 5min. The cells were resuspended in 0.25M-sucrose in 0.05M-Tris-HCl buffer, pH 7.5, containing 25mM-KCl and 0.5mM-MgCl₂, and homogenized under conditions described by Harwood *et al.* (1974d). Nuclei and mitochondria were sedimented at 4°C by centrifugation at 10000g (r_{av} , 6.5cm) for 10min in the 10 $\times$ 10ml titanium angle rotor of an MSE Superspeed 65 ultracentrifuge. The pellet was rehomogenized, centrifuged at 10000g for 10min and the two supernatants were combined. This post-mitochondrial fraction was layered over a discontinuous gradient consisting of 0.5M- and 2.0M-sucrose in 0.05M-Tris-HCl buffer, pH 7.5, containing 25mM-KCl and 0.5mM-MgCl₂, and centrifuged at 105000g for 21h (10 $\times$ 10ml titanium angle rotor of an MSE Superspeed 65 centrifuge) to sediment free ribosomes. The layer at the interface was collected and treated with 0.1% Triton X-100 and centrifuged at 105000g for 2h to pellet membrane-bound ribosomes (Blobel & Potter, 1967).

Sucrose-gradient-centrifugation profiles of the populations of free and membrane-bound ribosomes were obtained as described by Harwood *et al.* (1974c) and analysed at 254nm. Regions of the gradients were pooled as described in the Results section, and assayed for the presence of hydroxy[^{14}C]proline or hydroxy[^{14}C]lysine in the nascent polypeptides.

Hydroxy[^{14}C]proline was determined by a specific radiochemical assay procedure (Juva & Prockop, 1966) after hydrolysis of sample in 6M-HCl at 105°C for 24h. Determination of [^{14}C]lysine and hydroxy[^{14}C]lysine in samples was achieved by acid hydrolysis as above, followed by chromatography of the hydrolysates at pH 5.3 on the short column of a Jeol JLC-6AH amino acid analyser. Fractions (1.1ml) were collected and 0.5ml portions were counted for radioactivity in 10ml of scintillant [2 parts of toluene containing 5g of 2,5-diphenyl-oxazole/litre and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 1 part of Triton X-100] to which was added 0.5ml of water and 0.2ml of 2M-HCl to avoid precipitation of the sample.

Incubation of microsomal preparations with puromycin to test the directional discharge of procollagen polypeptides

Cells were pulse-labelled with [^{14}C]proline for 4min followed by isolation of microsomes (microsomal preparations) as described by Harwood *et al.* (1974d). The microsomal pellet was rinsed and resuspended in 2.0ml of the above sucrose-Tris-KCl-MgCl₂ buffer. The suspension was incubated with shaking at 37°C with appropriate cofactors including those for procollagen proline hydroxylase (EC 1.14.11.2) (Harwood *et al.*, 1974b). After a preincubation of 15min to permit the 'healing' of disrupted vesicles (Bevan, 1971) samples were

incubated for a further 10min with or without puromycin at a final concentration of 0.2mM. The samples were centrifuged at 105000g for 1 h at 4°C. The resultant supernatant (I), representing the incubation medium, was collected and the pellet rinsed and resuspended in 5 ml of phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 0.5% (v/v) Triton X-100 and 5mM-MgCl₂. The tubes were left for 15min at 4°C and then re-centrifuged at 105000g for 1 h. This supernatant (II), containing the contents of the microsomal vesicles, was collected together with the ribosomal pellet. The presence of hydroxy[¹⁴C]proline in supernatants (I) and (II) and the ribosomal pellet was determined after hydrolysis in 6M-HCl for 24 h at 105°C.

Gel filtration of ¹⁴C-labelled protein on sodium dodecyl sulphate-agarose

The extent of aggregation of the procollagen polypeptides resulting from disulphide bonding was examined in various cell fractions by gel filtration on sodium dodecyl sulphate-agarose before and after reduction with 2-mercaptoethanol. Samples containing ¹⁴C-labelled protein were heated at 100°C for 2min in 2ml of 0.02M-Tris-HCl buffer (pH 7.4) containing 1% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol. The samples were incubated for a further 2 h at 27°C before dialysis for 24 h at room temperature against two changes of 500ml of 0.1% sodium dodecyl sulphate in 0.02M-Tris-HCl buffer, pH 7.4. In the experiments where mercaptoethanol was omitted, the sample was made 0.1M in iodoacetamide and neutralized before being heated at 100°C for 2min in 1% sodium dodecyl sulphate. This inclusion of an alkylating reagent during the denaturation process was essential to avoid non-specific reduction of disulphide bonds, which is found to occur with some preparations of sodium dodecyl sulphate (Parkhouse, 1971).

The samples were centrifuged at 3000g for 10min to remove any small residue of insoluble material and then chromatographed at room temperature on a column of 6% agarose (Bio-Gel A-5m, 200-400 mesh) which was equilibrated with 0.02M-Tris-HCl buffer, pH 7.4, containing 0.1% sodium dodecyl sulphate and 0.02% sodium azide. The column was 1.5cm × approx. 95cm and fractions (2.4ml) were collected at a flow rate of approximately 5ml/h. The total elution volume (*V_t*) for each chromatogram was established by addition of 0.25 μCi of ³H₂O to the sample before chromatography.

The ¹⁴C in the fractions was assayed by taking 0.1 ml samples with 0.2ml of 2M-HCl in the solvent system for liquid-scintillation counting of radioactivity as described by Harwood *et al.* (1974c). Hydroxy-[¹⁴C]proline in the fractions was assayed as described above.

Results

Demonstration that procollagen is synthesized on large membrane-bound polyribosomes

To determine the site of synthesis of cartilage procollagen, cells were pulse-labelled for 4min with [¹⁴C]proline and free and membrane-bound ribosomes were isolated. The nascent polypeptide chains in the ribosomal pellets were analysed for the synthesis of hydroxy[¹⁴C]proline (Table 1). Approximately 90% of the hydroxy[¹⁴C]proline was found in polypeptides attached to membrane-bound ribosomes.

Since the synthesis of hydroxy[¹⁴C]lysine in these cells can also be used as an index of collagen synthesis a parallel experiment was carried out in which cells were labelled with [¹⁴C]lysine for 4min. Assays of free and membrane-bound ribosomes for hydroxy-[¹⁴C]lysine provided the first definitive evidence for the synthesis of hydroxylysine on nascent polypeptides and this process was shown to occur almost exclusively on polypeptides attached to membrane-bound ribosomes (Table 1).

An analysis of the size distribution of the polyribosomes present in the free and membrane-bound fractions isolated for cartilage cells is shown in Fig. 1. The profile of membrane-bound ribosomes reveals the presence of a population of large

Table 1. *Distribution of hydroxy[¹⁴C]proline and hydroxy-[¹⁴C]lysine synthesized on free and membrane-bound ribosomes of cartilage cells*

In Expt. 1 526 × 10⁶ cells were incubated in 10ml of modified Krebs medium at 37°C with 25 μCi of [¹⁴C]proline for 4min. The incubation was stopped by the addition of cycloheximide and the cells were collected by centrifugation, washed and homogenized. Free and membrane-bound ribosomes were isolated and a sample was assayed for hydroxy[¹⁴C]proline as described in the text. In Expt. 2 718 × 10⁶ cells were pulse-labelled for 4min with 50 μCi of [¹⁴C]lysine and then free and membrane-bound ribosomes were isolated and analysed for hydroxy[¹⁴C]lysine.

	Free ribosomes	Bound ribosomes
Expt. 1 Total [¹⁴ C]proline incorporated (d.p.m.)	17871	33860
Hydroxy[¹⁴ C]proline synthesized (d.p.m.)	462	3625
Distribution of hydroxy- [¹⁴ C]proline (%)	11.3	88.7
Expt. 2 Total [¹⁴ C]lysine incorporated (d.p.m.)	21721	43080
Hydroxy[¹⁴ C]lysine synthesized (d.p.m.)	24	3120
Distribution of hydroxy- [¹⁴ C]lysine (%)	0.8	99.2

Fig. 1. Density-gradient profiles of free and membrane-bound ribosomes from cartilage cells

Cartilage cells (8×10^8) were homogenized and free and membrane-bound ribosomes were isolated as described in the text. The pellets of free and bound ribosomes were resuspended in 0.02M-Tris-HCl buffer (pH 7.4) containing 0.24M-KCl and 7.5mM-MgCl₂ and portions were layered on 4.5ml linear 15–40% (w/v) sucrose density gradients prepared in the same buffer. These gradients were then centrifuged at 112000g (r_{av} 7.4cm) for 1h at 4°C in the 3×5 ml aluminium swing-out rotor of an MSE Superspeed 65 ultracentrifuge. Calibration of the sucrose gradients and determination of the absorbance at 254nm were carried out as described by Harwood *et al.* (1974c). —, Membrane-bound ribosomes; ----, free ribosomes.

Table 2. Distribution of hydroxy[¹⁴C]proline and hydroxy[¹⁴C]lysine in sucrose-density-gradient profiles of membrane-bound ribosomes from cartilage cells

The membrane-bound ribosomes isolated in the experiments described in Table 1 from cells pulse-labelled with [¹⁴C]proline and [¹⁴C]lysine were analysed by sucrose-gradient centrifugation. Regions of the gradient were pooled to yield fraction (I) containing single ribosomes, fraction (II) containing light and medium polyribosomes (<250S), and fraction (III) containing heavy polyribosomes (>250S). Each fraction was assayed for hydroxy[¹⁴C]proline and hydroxy[¹⁴C]lysine in the respective experiments.

	Radioactivity (d.p.m.)		
	Fraction (I)	Fraction (II)	Fraction (III)
Expt. 1 Total [¹⁴ C]proline	6273	18723	27924
Hydroxy[¹⁴ C]proline	112	527	3101
Expt. 2 Total [¹⁴ C]lysine	7931	25642	34786
Hydroxy[¹⁴ C]lysine	20	409	5629

polyribosomes having a sedimentation coefficient greater than 250S. This observation is consistent with previous studies, which indicated that collagen synthesis occurs on polyribosomes of the size range 300–350S (Lazarides & Lukens, 1971; Harwood *et al.*, 1974a,c). To determine more precisely the size of polyribosomes on which cartilage procollagen synthesis occurs, membrane-bound

ribosomes from cells labelled with [¹⁴C]proline for 4min were analysed by sucrose-density-gradient centrifugation. The isolated ribosomes were pooled to yield fraction (I) containing single ribosomes, fraction (II) containing light and medium polyribosomes (<250S) and fraction (III) containing heavy polyribosomes (>250S). Each fraction was assayed for the presence of hydroxy[¹⁴C]proline and the results shown in Table 2 indicate that the biosynthesis of cartilage procollagen occurs on heavy membrane-bound polyribosomes. In an analogous experiment, membrane-bound ribosomes from cells labelled with [¹⁴C]lysine were centrifuged on sucrose density gradients and similar fractions pooled. The results of assays for hydroxy[¹⁴C]lysine present in the fractions are shown in Table 2 and confirm that cartilage procollagen is synthesized on large membrane-bound ribosomal aggregates.

Demonstration of the vectorial release of procollagen polypeptides into the lumen of the endoplasmic reticulum

The direction of discharge of newly synthesized procollagen polypeptides was examined after their puromycin-induced release from membrane-bound ribosomes. Microsomes isolated from cells labelled for 4min with [¹⁴C]proline were incubated in the presence and absence of puromycin under the conditions described by Harwood *et al.* (1974b). After incubation with puromycin less than 15% of total ¹⁴C and less than 5% of hydroxy[¹⁴C]proline were released into supernatant (I) which is equivalent to the cytoplasm in the cell (Table 3). Of the ¹⁴C label that remained associated with the microsomal pellet, approx. 32% was released by treatment with Triton X-100 compared with less than 18% in the control sample. Analyses of supernatant (II) from test and control samples for hydroxy[¹⁴C]proline indicated that approx. 40% of the puromycin–procollagen peptides were released by detergent action compared with less than 20% released in the control.

Determination of the site and extent of inter-chain disulphide-bond formation

The extent of disulphide bonding of the procollagen secreted by cartilage cells was analysed by chromatography of unreduced and reduced samples of the medium after incubation of cells for 2h with [¹⁴C]proline. Earlier studies with cells isolated by enzymic digestion (Uitto *et al.*, 1972; Grant *et al.*, 1972b, 1973; Dehm & Prockop, 1973; Harwood *et al.*, 1973) have indicated that some degradation of secreted material may occur unless appropriate precautions are taken and for this reason incubation of the cartilage cells was carried out in the presence of

Table 3. *Effect of puromycin on the release of total ^{14}C and hydroxy[^{14}C]proline from microsomes obtained from cartilage cells pulse-labelled for 4 min with [^{14}C]proline*

Cartilage cells (823×10^6) were incubated with $50 \mu\text{Ci}$ of [^{14}C]proline in 15 ml of modified Krebs medium at 37°C for 4 min. Microsomes were isolated by differential centrifugation and portions were incubated with and without puromycin under the conditions described by Harwood *et al.* (1974b), which included the cofactors for procollagen proline hydroxylase.

Assay	Incubation conditions	$10^{-3} \times$ Total radio-activity (d.p.m.)	Radioactivity in supernatant (I) (%)	Radioactivity in supernatant (II) (%)	Radioactivity in the ribosomal pellet (%)
Total ^{14}C	Control	107.6	5.9	17.8	76.3
	+Puromycin	89.7	14.7	32.1	53.2
Hydroxy[^{14}C]proline	Control	12.6	2.9	16.4	80.7
	+Puromycin	11.9	4.9	36.6	55.5

Fig. 2. *Gel filtration on sodium dodecyl sulphate-agarose of extracellular and intracellular proteins synthesized by cartilage cells incubated for 2 h with [^{14}C]proline*

Duplicate samples of 150×10^6 cartilage cells in 15 ml of modified Krebs medium containing 20% (v/v) of dialysed foetal bovine serum were incubated with $10 \mu\text{Ci}$ of [^{14}C]proline for 2 h at 37°C . Cells and medium were separated by centrifugation and subjected to gel filtration on sodium dodecyl sulphate-agarose with and without prior reduction with 2-mercaptoethanol. (a) A 2 ml sample of medium was alkylated and denatured in sodium dodecyl sulphate (1%, w/v) as described in the text. The sample was dialysed against column buffer before chromatography. Fractions (2.4 ml) were collected and the void volume (V_0) of the column was 60 ml (fraction 25) and the total volume (V_t) was 173 ml (fraction 72). A portion (0.2 ml) of each fraction was taken for determination of total ^{14}C and the remainder was hydrolysed in 6M-HCl at 105°C for 24 h before being assayed for hydroxy[^{14}C]proline. (b) A 2 ml sample of medium was reduced with 2-mercaptoethanol and denatured in sodium dodecyl sulphate. The sample was dialysed and chromatographed as described above. (c) Gel filtration of ^{14}C -labelled protein of the cell pellet after alkylation and denaturation. (d) Gel filtration of ^{14}C -labelled protein of the cell pellet after reduction and denaturation. ●, Elution of total ^{14}C ; ○, elution of hydroxy[^{14}C]proline.

20% (v/v) foetal calf serum that had been extensively dialysed against the modified Krebs medium at 4°C .

The results shown in Fig. 2(a) indicate that when medium was alkylated, sodium dodecyl sulphate-treated and chromatographed on sodium dodecyl

sulphate-agarose without prior reduction, over 90% of the hydroxy[^{14}C]proline-containing polypeptides were eluted near the void volume of the column corresponding to a molecular weight in excess of 300 000. When medium was treated with 2-mercapto-

ethanol before chromatography the hydroxy[^{14}C]proline-containing polypeptides now eluted in a position corresponding to individual pro- α chains having a molecular weight of approx. 125000 (Fig. 2b).

In contrast with the above finding that almost all the secreted procollagen is present in a disulphide-bonded form, similar studies carried out on the cartilage cells labelled for 2h with [^{14}C]proline indicated that only about 25% of intracellular procollagen polypeptides were disulphide bonded (Figs. 2c,d). This observation suggests that disulphide bonding is an intracellular event and a study of the extent of inter-chain disulphide-bond formation in various subcellular fractions was undertaken to establish the site of this process.

When rough endoplasmic reticulum was isolated from cartilage cells labelled for 2h with [^{14}C]proline and a sample submitted to gel filtration without

reduction (Fig. 3a), approximately 20% of the hydroxy[^{14}C]proline-containing polypeptides chromatographed in a peak near the void volume. A second sample was reduced with mercaptoethanol, and on subsequent chromatography the majority of the hydroxy[^{14}C]proline was eluted in the position of individual pro- α chains (Fig. 3b), indicating that the aggregated material observed in the untreated sample consisted of disulphide-bonded pro- α chains. Isolation of smooth endoplasmic reticulum from these cells and subsequent gel filtration without reduction showed that about 85% of hydroxy[^{14}C]proline synthesized was present in high-molecular-weight aggregates (Fig. 3c). That this material consisted of disulphide-bonded cartilage procollagen polypeptides was indicated by chromatography of a reduced sample of 'smooth' microsomes when the majority of the hydroxy[^{14}C]proline was eluted in a new peak representative of pro- α chains (Fig. 3d).

Fig. 3. Gel filtration on sodium dodecyl sulphate-agarose of ^{14}C -labelled protein of submicrosomal fractions isolated from cartilage cells labelled for 2h with [^{14}C]proline

Duplicate samples of 340×10^6 cartilage cells were incubated in 34 ml of modified Krebs medium at 37°C with $25 \mu\text{Ci}$ of [^{14}C]proline. The cells were collected by centrifugation, homogenized and fractionated to obtain fractions of rough and smooth endoplasmic reticulum as described previously (Harwood *et al.*, 1974d). The submicrosomal fractions from one incubation were alkylated and denatured with iodoacetamide and sodium dodecyl sulphate and those from the duplicate incubation were reduced and denatured with 2-mercaptoethanol and sodium dodecyl sulphate as described in the text. Only one-half of each sample of rough endoplasmic reticulum was applied to the column whereas all of the fractions of smooth endoplasmic reticulum were chromatographed. The chromatographic conditions were the same as described in Fig. 2. (a) Sample of un-reduced rough endoplasmic reticulum. (b) Sample of reduced rough endoplasmic reticulum. (c) Un-reduced smooth endoplasmic reticulum. (d) Reduced smooth endoplasmic reticulum. ●, Elution of total ^{14}C ; ○, elution of hydroxy[^{14}C]proline.

These results suggest that disulphide bonding between cartilage pro- α chains begins in the rough endoplasmic reticulum and to assess whether any disulphide bonding occurs on ribosomes, cartilage cells were pulse-labelled for 4 min with [^{14}C]proline, the 'rough' microsomes isolated and chromatographed on sodium dodecyl sulphate-agarose without mercaptoethanol treatment. Hydroxy[^{14}C]proline was detected only in polypeptides eluted in positions corresponding to individual pro- α chains or incomplete procollagen polypeptides (Fig. 4). Since the time required to synthesize the complete cartilage collagen pro- α chain is approx. 6–7 min (Miller *et al.*, 1973) the major proportion of label incorporated in this experiment is seen to be in recently completed and nascent polypeptides. The failure to observe any label in material larger than individual pro- α chains indicates the absence of disulphide-bonded aggregates and demonstrates that disulphide bonding is a post-ribosomal event in embryonic chick cartilage cells. The above data correlate well with previous studies on inter-chain disulphide-bond formation in the synthesis of tendon procollagen (Harwood *et al.*, 1973; Schofield *et al.*, 1974). In both cell types disulphide-bond formation is seen to occur in the rough endoplasmic reticulum after release of the completed chains from membrane-bound ribosomes. However, studies

of the extent of intracellular disulphide bonding under steady-state conditions (i.e. a 2 h labelling period) suggest that the rates of inter-chain disulphide-bond formation differ since in tendon cells 45–60% of procollagen polypeptides are disulphide linked (Harwood *et al.*, 1973; Schofield *et al.*, 1974) whereas the above studies indicate that approx. 25% of intracellular cartilage procollagen polypeptides are disulphide bonded. This latter observation has been confirmed in similar studies reported by Uitto & Prockop (1974d).

To investigate further the intracellular processing of cartilage procollagen, cells were pulse-labelled with [^{14}C]proline for 4 min followed by a 12 min chase period with unlabelled proline. Rough- and smooth-endoplasmic-reticulum fractions were separated as

Fig. 4. Gel filtration on sodium dodecyl sulphate-agarose of unreduced ^{14}C -labelled protein of the rough-endoplasmic-reticulum fraction from cells labelled for 4 min with [^{14}C]proline

Some 736×10^6 cells were incubated in 15 ml of modified Krebs medium at 37°C with $50 \mu\text{Ci}$ of [^{14}C]proline. After 4 min the incubation mixture was cooled to 4°C , protein synthesis was inhibited by the addition of 1.6 ml of cycloheximide (1 mg/ml) and the sample was made 0.25 M in sucrose. Rough endoplasmic reticulum was isolated as described in the text, alkylated and treated with sodium dodecyl sulphate before chromatography under the conditions described in Fig. 2. ●, Elution of total ^{14}C ; ○, elution of hydroxy[^{14}C]proline.

Fig. 5. Gel filtration on sodium dodecyl sulphate-agarose of unreduced ^{14}C -labelled protein of submicrosomal fractions from cells labelled with [^{14}C]proline for 4 min followed by a chase with unlabelled proline for a further 12 min

Some 650×10^6 cells were incubated in 15 ml of modified Krebs medium at 37°C with $50 \mu\text{Ci}$ of [^{14}C]proline. After 4 min unlabelled proline was added to a final concentration of $100 \mu\text{g/ml}$ and the incubation continued for a further 12 min. Rough and smooth endoplasmic reticulum were isolated, alkylated and treated with sodium dodecyl sulphate before chromatography under the conditions described in Fig. 2. (a) Unreduced rough endoplasmic reticulum. (b) Unreduced smooth endoplasmic reticulum. ●, Elution of total ^{14}C ; ○, elution of hydroxy[^{14}C]proline.

described above and samples were chromatographed on sodium dodecyl sulphate-agarose without reduction. Although the majority of the radioactivity was still present in the 'rough' microsomes only 26% of the hydroxy[^{14}C]proline label in this fraction eluted in the position of disulphide-bonded aggregates and the majority of the hydroxy[^{14}C]proline was present in polypeptides of the same size as pro- α chains (Fig. 5a). In contrast all the hydroxy[^{14}C]proline-containing polypeptides in the smooth-endoplasmic-reticulum fraction were present in material that eluted near the void volume and represented the disulphide-bonded cartilage procollagen (Fig. 5b). This experiment confirms that disulphide bonding of cartilage procollagen polypeptides commences in the rough endoplasmic reticulum. The presence of fully disulphide-bonded procollagen in the smooth endoplasmic reticulum suggests that either disulphide bonding is completed in this cellular compartment or that procollagen needs to be in a disulphide-bonded form to be transferred to this region of the endoplasmic reticulum.

Discussion

Studies on the fractionation of matrix-free tendon and cartilage cells (Harwood *et al.*, 1974d) have suggested that it is possible to obtain subcellular fractions of a purity comparable with those obtained from tissues such as liver and kidney. This approach has enabled us to localize the hydroxylases (Harwood *et al.*, 1974d) and glycosyl transferases (Harwood *et al.*, 1975) which effect the post-translational modifications of the procollagen polypeptides. In the present study with cartilage cells we have determined the intracellular sites of procollagen messenger RNA translation, of hydroxyproline and hydroxylysine synthesis, and of pro- α chain association through disulphide bonds.

The results in Table 1 demonstrate that the assembly of cartilage pro- α chains takes place on membrane-bound ribosomes. This observation is consistent with earlier reports on the synthesis of Type I procollagen whose assembly also occurs on bound ribosomes (Diegelmann *et al.*, 1973; Harwood *et al.*, 1973; Burns *et al.*, 1974). In addition these studies confirm that the hydroxylation of peptidyl-proline is a ribosomal event (Miller & Udenfriend, 1970; Lazarides *et al.*, 1971; Harwood *et al.*, 1973; Uitto & Prockop, 1974c) and for the first time direct evidence is presented that the hydroxylation of peptidyl-lysine also commences while the growing chain is still attached to the ribosome. It should be noted that indirect evidence for the synthesis of hydroxylysine on nascent chains has recently been provided by short-term pulse-labelling experiments with chick tendon cells (Uitto & Prockop, 1974c). The fact that procollagen

proline hydroxylase (Guzman & Cutroneo, 1973; Diegelmann *et al.*, 1973; Olsen *et al.*, 1973; Harwood *et al.*, 1973), and procollagen lysine hydroxylase (Harwood *et al.*, 1974d) have been shown to be microsomal enzymes and associated almost exclusively with rough endoplasmic reticulum obviously correlates well with the above findings.

Examination by sucrose-density-gradient centrifugation of the size of membrane-bound ribosomes on which hydroxyproline and hydroxylysine synthesis occurs demonstrated that the nascent procollagen polypeptides were associated with polyribosomes of sedimentation coefficient in the range 250–400S (Table 2). These results are consistent with the synthesis of cartilage pro- α chains being directed by individual monocistronic messenger RNA species, as has been found with other types of procollagen (Goldberg & Green, 1967; Lazarides & Lukens, 1971; Kerwar *et al.*, 1973; Harwood *et al.*, 1974a).

The demonstration that cartilage procollagen, like other secreted proteins (Campbell, 1971), is synthesized on membrane-bound polyribosomes suggests that its route of secretion is likely to follow the classical pathway via the cisternae of the endoplasmic reticulum and the Golgi apparatus. However, earlier studies based on morphological and electron microscope radioautographic techniques provided conflicting information on the route of collagen secretion (Sheldon & Kimball, 1962; Revel & Hay, 1963; Ross & Benditt, 1965; Cooper & Prockop, 1968; Salpeter, 1968; Reith, 1968) with some evidence suggesting that cartilage collagen was transported to the plasma membrane via the cytoplasm (Cooper & Prockop, 1968; Reith, 1968; Salpeter, 1968). However, more recent studies using immunological (Olsen & Prockop, 1974) and biochemical (Harwood *et al.*, 1974b) techniques to examine the secretion of procollagen by embryonic chick tendon cells suggest that the route of procollagen secretion is in fact through the cisternae of the endoplasmic reticulum and the Golgi apparatus.

The study reported here indicates that when nascent cartilage procollagen polypeptides are prematurely released by puromycin they are transferred from the ribosomes across the membrane into the microsomal vesicles. The results shown in Table 3 are very similar to those reported in studies of the puromycin-induced release of tendon procollagen (Harwood *et al.*, 1974b) and other secreted proteins (Redman *et al.*, 1966; Redman, 1969; Bevan, 1971). Since the content of the microsomal vesicles is the equivalent of the intracisternal space in the cell these results provide evidence that cartilage procollagen polypeptides follow the same secretory route as described for albumin, serum glycoproteins, etc. [for reviews, see Jamieson (1972) and Grant *et al.* (1975)]. The possibility that puromycin-collagen peptides differ in their mode of secretion from

normally released collagen molecules was raised by studies with puromycin-treated embryonic chick tibiae (Bhatnagar *et al.*, 1967). However, the data obtained for the puromycin release of nascent tendon procollagen polypeptides were confirmed by studies of the susceptibility of the nascent procollagen chains to limited proteolysis (Harwood *et al.*, 1974b). Thus it appears that studies using puromycin-induced release yield results that are likely to reflect the normal direction of discharge in the cell.

The demonstration that, like the more studied Type I procollagens, the procollagen secreted by matrix-free cartilage cells contains cysteine (Dehm & Prockop, 1973) indicated the likelihood that the amino-terminal extensions of cartilage procollagen participate in chain association through disulphide bonds as found for procollagen synthesized by tendon cells (Dehm *et al.*, 1972; Harwood *et al.*, 1973; Schofield *et al.*, 1974), calvaria (Monson & Bornstein, 1973; Fessler *et al.*, 1973) and cultured skin fibroblasts (Burgeson *et al.*, 1972; Goldberg & Sherr, 1973; Sherr *et al.*, 1973). The results in Fig. 2 demonstrate that in extracellular cartilage procollagen the individual pro- α chains are linked by disulphide bonds and that disulphide-bond formation occurs before secretion. Further studies were directed to ascertain the subcellular location of this process.

The 4min pulse-labelling experiment described in Fig. 4 indicates that the formation of inter-chain disulphide bonds is a post-ribosomal event but since there is good evidence that large polypeptides can begin to assume secondary and tertiary structure before release of the completed chain from the ribosome (Hamlin & Zabin, 1972) the possibility that intra-chain disulphide-bond formation within pro- α chains (Furthmayr *et al.*, 1972) may occur at this stage cannot be excluded. This observation agrees with previous studies on the intracellular disulphide bonding of tendon procollagen (Harwood *et al.*, 1973; Schofield *et al.*, 1974) and contrasts with studies on the synthesis *in vitro* of immunoglobulin G which have indicated that active antibody molecules comprising disulphide-linked H and L chain complexes may be formed on the ribosome while at least one of the polypeptide chains is still being synthesized (Askonas & Williamson, 1967; Schubert, 1968).

The presence of disulphide-bonded aggregates within the rough endoplasmic reticulum in the pulse-chase experiment (Fig. 5a) suggests that disulphide bonding occurs soon after release of completed pro- α chains. After the 16min period of this experiment less than 10% of the hydroxy[14 C]-proline in the submicrosomal fractions is associated with the smooth endoplasmic reticulum and over 90% with the rough endoplasmic reticulum. Only approximately 20% of the pro- α chains in this latter fraction are linked by inter-chain disulphide

bonds. In similar pulse-chase experiments conducted with chick tendon cells it was reported that, after a 4min pulse-label with [14 C]proline followed by a 9min chase with unlabelled proline, approximately 60% of intracellular pro- α chains were present as disulphide-linked aggregates (Schofield *et al.*, 1974). Thus it appears that the rate of chain association through disulphide bonds is significantly slower in cartilage cells than the rate reported for this process in tendon cells. This conclusion is supported by the observation that different proportions of pro- α chains are present as disulphide-linked aggregates in tendon and cartilage cells labelled under steady-state conditions (see above). In addition, a preliminary report of studies with intact cartilage cells to assess the rate of disulphide bonding of intracellular procollagen polypeptides by pulse-chase experiments, suggests that this process is slower in cartilage than in tendon cells (Uitto & Prockop, 1974d).

Since there is evidence that the alignment of pro- α chains and formation of the triple helix may be facilitated by the prior formation of inter-chain-disulphide bonds, a delay in disulphide-bond formation is likely to result in a delay in helix formation (Uitto & Prockop, 1973; Schofield *et al.*, 1974). Recent work has indicated that the hydroxylation of procollagen requires that the polypeptides are in a non-helical conformation (Berg & Prockop, 1973; Murphy & Rosenbloom, 1973; Ryhänen & Kivirikko, 1974) and hence the delay in disulphide-bond formation in cartilage cells may explain the more extensive secondary modifications of cartilage procollagen as compared with tendon procollagen. Thus it would appear that disulphide-bond formation may be an important point of control in the biosynthesis of procollagen and the variations in the timing of this process in different cell types may be related to the differences observed in the times required for the synthesis and secretion of procollagen by tendon (Dehm & Prockop, 1972), cartilage (Dehm & Prockop, 1973), artery (Schofield & Harwood, 1975) and lens cells (Grant *et al.*, 1972a).

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