Synthesis of Elastin in Aortas from Chick Embryos. Conversion of Newly Secreted Elastin to Cross-Linked Elastin without Apparent Proteolysis of the Molecule†

Giorgio M. Bressan‡ and Darwin J. Prockop*

ABSTRACT: The biosynthesis of elastin was examined in matrix-free cells isolated by enzymic digestion of aortas from 17-day old chick embryos. After the cells were incubated with [^{14}C]proline and then were rapidly boiled in buffer containing high concentrations of protease inhibitors and sodium dodecyl sulfate, about one-quarter of the intracellular ^{14}C-labeled protein was recovered as an elastin component with an apparent molecular weight of about 72 000. Examination of the medium from the cell suspension indicated that the largest elastin component secreted by the cells also had an apparent molecular weight of about 72 000. Pulse-chase experiments with intact aortas demonstrated that about two-thirds of the 72 000-dalton component disappeared in 2 h, apparently because it was converted to cross-linked fibers. When cross-linking was inhibited with penicillamine, the 72 000-dalton component persisted in the tissue 5 h. When cross-linking was inhibited with β-aminopropionitrile, the elastin component of 72 000 daltons persisted for about 2 h, but thereafter it was gradually degraded to small peptides which were recovered in the incubation medium. The results suggest that elastin is secreted by cells in chick aorta as a polypeptide of about 72 000 daltons and that the secreted protein is incorporated into elastin fibers without cleavage to a protein of considerably smaller size.

Several proteins synthesized for "export" from cells are first assembled and secreted as large precursor forms which must undergo proteolysis in order to be converted to functional proteins. The list of such proteins includes several peptide hormones (for review, see Steiner et al., 1974), a number of digestive enzymes (see Desnuelle and Rovery, 1961), and procollagen (see Prockop et al., 1976). Elastin is found in tissues as an insoluble, highly cross-linked fiber, and several laboratories have demonstrated that elastin components which are apparently precursors of insoluble fibers can be extracted from tissues and cell cultures synthesizing elastin (Sandberg et al., 1969; Smith et al., 1972; Abraham et al., 1974; Sykes and Partridge, 1974; Foster et al., 1975; Rucker et al., 1975a). Since extractable elastin components with apparent molecular weights of either 57 000 or 72 000 were obtained under different experimental conditions, it has been suggested that the elastin component with a molecular weight of about 72 000 may be a precursor which is cleaved to a form with a molecular weight of 57 000 before it is assembled into elastin fibers (Foster et al., 1975).

In the work presented here, we have explored the hypothesis that elastin is synthesized as a precursor form by examining the intracellular elastin in matrix-free cells prepared from aortas and associated blood vessels of chick embryos. Also, we have followed the conversion of newly synthesized elastin to cross-linked elastin in aortas from chick embryos.

Materials and Methods

Materials. Elastin solubilized from the aortas of copper-deficient chicks (Rucker et al., 1975a) was a generous gift from Dr. Robert B. Rucker, Department of Nutrition and Food Science Technology, University of California, Davis, Calif. Proteins which were used as standards for estimating the size of unknown components were obtained from Boehringen (Mannheim). $NaDodSO_4$[1] (electrophoresis grade) was purchased from Bio-Rad Laboratories (Richmond, Calif.). β-APN was purchased from General Biochemicals (Chagrin Falls, Ohio), and Nonidet P40 was purchased from Shell Chemicals. Other materials were obtained from suppliers used previously (Uitto et al., 1976; Olsen et al., 1976).

Isolation and Incubation of Matrix-Free Cells from Embryonic Aortas. About 5×10^8 cells were isolated by controlled enzymic digestion of aortas and associated large blood vessels from one hundred 17-day old chick embryos as described previously (Uitto et al., 1976). The cells were washed in modified Krebs medium containing 10% fetal calf serum and then they were incubated in modified Krebs medium without fetal calf serum. To increase the total amount of ^{14}C-labeled protein recovered within the cells (see below), 1 μM colchicine was added to the medium and the cells were preincubated for 15 min in medium containing 1 μM colchicine before addition of [^{14}C]proline. After the incubation, the cells were removed by centrifuging at 1300*g* for 6 min at room temperature. The cell pellet was homogenized in 5 mL of a "standard homogenizing buffer" which contained 0.02 M sodium phosphate (pH 7.4), 0.1 M NaCl, a mixture of protease inhibitors (Olsen et al., 1976), 1 mg/mL of penicillamine (Sigma Chemical Co.), 0.1 mg/mL of β-APN, and 0.1% Nonidet P40. Homogenization was carried out in a Teflon and glass homogenizer with 10 strokes at full speed with a constant torque motor (Schwaben Präzision, Nördlingen, Germany).

Isolation and Incubation of Aortas. Aortas and associated large blood vessels were dissected out of 17-day old chick embryos as described previously (Uitto et al., 1976), washed

† From the Department of Biochemistry, College of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, New Jersey, 08854. *Received September 21, 1976.* This work was supported in part by Research Grant AM16516 from the National Institutes of Health.

‡ Present address: Instituto di Istologia ed Embriologia dell' Universita di Padova, Padova, Italy.

[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; β-APN, β-aminopropionitrile fumarate; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; PAGE, polyacrylamide gel electrophoresis.

in modified Krebs medium without fetal calf serum, and incubated in modified Krebs medium without fetal calf serum. In most experiments, the aortas and associated blood vessels from ten chick embryos were incubated in about 3 mL of medium containing 3 or 5 μCi of [^{14}C]proline. After the incubation, the tissues were blotted dry on filter paper and stored frozen prior to further processing. In experiments in which the ^{14}C label was chased, the tissues were removed from the medium used for labeling with [^{14}C]proline, blotted on filter paper, and transferred to fresh medium containing 500 μg/mL of [^{12}C]proline.

Samples containing the blood vessels from ten chick embryos were homogenized in the standard homogenizing buffer and under the same conditions used to homogenize matrix-free cells except that the number of strokes was increased to 30.

Extraction of ^{14}C-Labeled Protein from Matrix-Free Cells with NaDodSO$_4$. Matrix-free cells were incubated with [^{14}C]proline as described above and the cell suspension was centrifuged at 1300*g* for 6 min at room temperature. The pellet from about 1.5×10^8 cells was then extracted with NaDodSO$_4$. In one series of experiments the cell pellet was homogenized in 5 mL of the standard homogenizing buffer as described above. The homogenate was centrifuged at 20 000*g* for 20 min at 4 °C, and the supernatant fraction was lyophilized. The sample was then rapidly dissolved at 100 °C in 3 mL of 5% NaDodSO$_4$ in 0.01 M sodium phosphate buffer, pH 7.4. The NaDodSO$_4$ solution was preheated to 100 °C and the sample was heated at 100 °C for 5 min immediately after addition of the NaDodSO$_4$. The sample was dialyzed at room temperature against 1% NaDodSO$_4$ in 0.01 M sodium phosphate buffer, pH 7.4, and it was examined by gel filtration in NaDodSO$_4$ as described below. In a second series of experiments the cell pellet was directly dissolved at 100 °C in 3 mL of buffer containing 5% NaDodSO$_4$ and protease inhibitors. The buffer solution contained: 5% NaDodSO$_4$; 0.01 M sodium phosphate, pH 7.4; 5 mM EDTA; 0.2 mM phenylmethanesulfonyl fluoride; 5 mM *N*-ethylmaleimide; and 1 mM *p*-aminobenzamidine. The buffer was preheated to 100 °C and after the buffer was added to the cell pellet, the sample was immediately heated at 100 °C for 15 min. The sample was centrifuged at 20 000*g* for 20 min and the supernatant fraction was chromatographed on a gel filtration column (see below) which was equilibrated and eluted with 0.01 M sodium phosphate buffer, pH 7.4, containing 1% NaDodSO$_4$, 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 5 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzamidine, and 0.02% sodium azide.

Isolation of Elastin by Treatment of Cells or Tissue with Propanol–Butanol. Propanol–butanol extraction of elastin was carried out with minor modifications of the procedure of Sandberg et al. (1975). Samples of cells or tissues were homogenized in the standard homogenizing buffer as described above, and the homogenates were centrifuged at 20 000*g* for 20 min. The supernate fractions were lyophilized and then dissolved in 4 mL of 8 M urea at 4 °C. The sample was dialyzed overnight against 0.1 M ammonium formate, pH 4.5, and the ammonium formate concentration was adjusted to 0.5 M by adding the solid. The sample, at a pH of 5.2, was treated at 4 °C with propanol–butanol by dropwise addition first of 1.5 volumes of 1-propanol and then 2.5 volumes of 1-butanol. The white precipitate which formed was removed by centrifugation at 10 000*g* for 10 min. The supernate was evaporated in a rotary evaporator at 30 °C and then the sample was extracted five times at room temperature with 0.5 mL of 1% NaDodSO$_4$ in 0.01 M sodium phosphate buffer, pH 7.4. The combined extracts were dialyzed against the same buffer and taken for examination by gel filtration or electrophoresis in NaDodSO$_4$.

CNBr Digestion of Fractions from Cells and Tissues. CNBr digestion was carried out on several types of samples: ammonium sulfate precipitates of cells homogenized in the standard homogenizing buffer, pellets obtained by centrifuging cell or tissue homogenates at 20 000*g* for 20 min, and the protein precipitates which formed when the 20 000*g* supernatant fraction from cell or tissue homogenates were treated with propanol–butanol (see above). Each sample was dissolved in 2 to 3 mL of 88% formic acid and then 200 to 400 mg of CNBr was added (Rasmussen et al., 1975). The CNBr digestion was carried out for 24 h at room temperature. The CNBr was removed first by evacuating with a water pump and then by evaporating on a rotary evaporator at 30 °C. The residue was extracted five times at room temperature with 0.5 mL of 1% NaDodSO$_4$ in 0.01 M sodium phosphate buffer, pH 7.4.

Gel Filtration in NaDodSO$_4$. Gel filtration in NaDodSO$_4$ was carried out with minor modifications of the procedures employed previously (Uitto et al., 1976). A 1.5 × 85 cm column of 6% agarose (Bio-Gel A-5m, 200–400 mesh, Bio-Rad) was employed, and the column was equilibrated and eluted with 1% NaDodSO$_4$ in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.02% sodium azide. In some experiments, the eluting buffer also contained protease inhibitors (see above). Fractions of 2 mL were collected and aliquots of 0.2 mL were assayed in a liquid scintillation counter.

Polyacrylamide Gel Electrophoresis in NaDodSO$_4$. Electrophoresis in NaDodSO$_4$ was carried out using the buffer system described by King and Laemmli (1971) and the slab gel electrophoresis cell described by Studier (1973). The separating gel was prepared with 10% polyacrylamide. A stacking gel of 6% polyacrylamide was generally employed but the stacking gel was omitted in experiments designed to estimate molecular weight (Weber et al., 1972). All samples were dialyzed against a "sample buffer" as described by Olsen et al. (1976) and heated at 100 °C for 3 min before application to the gel. To examine elastin from the aortas of copper-deficient chicks, the gels were stained with Coomassie blue (Hoffmann et al., 1976). To prepare autoradiographs of ^{14}C-labeled elastin components, the gels were treated according to the method of Bonner and Laskey (1974), and the x-ray film was preflashed according to Laskey and Mills (1975). Both the stained gels and the autoradiographs were scanned by attaching 6-mm-wide strips of gel or x-ray film to the wall of a 10-cm-long cuvette and scanning the strips with a Gilford UV spectrophotometer and a linear transport accessory designed for scanning cylindrical gels. Control experiments demonstrated that the response of the x-ray film was linear under the conditions employed here.

Results

Isolation of Intracellular Elastin from Matrix-Free Cells. Matrix-free cells were isolated by enzymic digestion of the large blood vessels of chick embryos (Uitto et al., 1976) with essentially the procedures which were used to prepare matrix-free cells from tendon and cartilage (Dehm and Prockop, 1971, 1973). To identify the major elastin component in the matrix-free cells, about 5×10^8 cells were incubated in 70 mL of medium with 30 μCi of [^{14}C]proline for 3 h, and the total intracellular ^{14}C-labeled proteins were extracted with NaDodSO$_4$. Because the amount of intracellular ^{14}C-labeled protein was relatively small, 1 μM colchicine was added to the incubation medium in order to delay the secretion of both procollagen and the elastin component (Uitto et al., 1976).

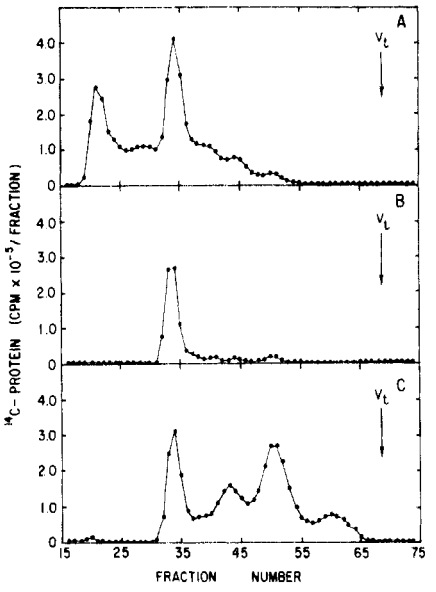

FIGURE 1: Gel filtration in $NaDodSO_4$ of ^{14}C-labeled protein isolated from matrix-free cells. About 5×10^8 cells were incubated for 3 h with 30 μCi of [^{14}C]proline and 1 μM colchicine. The cells were then homogenized in the "standard homogenizing buffer" and the homogenates were extracted as described in text. The column was prepared with 6% agarose and it was eluted with 1% $NaDodSO_4$ in 0.01 M sodium phosphate, pH 7.4 (see Materials and Methods). The void volume was in about fraction 20 and the total volume (V_t) in fractions 68–69. (A) ^{14}C-labeled protein extracted from homogenate with $NaDodSO_4$. (B) ^{14}C-labeled protein extracted from homogenate with propanol–butanol. (C) ^{14}C-labeled protein obtained after CNBr digestion of cell homogenates.

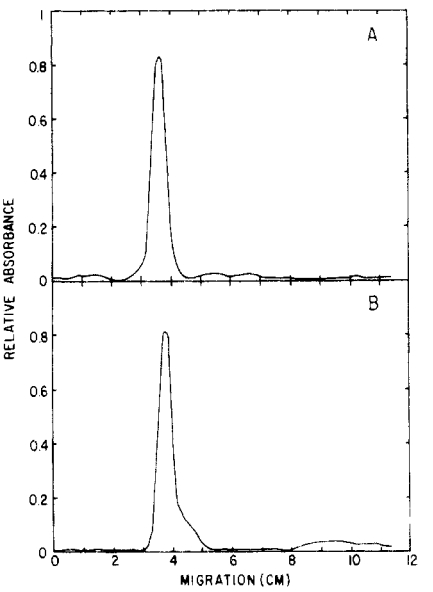

FIGURE 2: Electrophoresis in $NaDodSO_4$ of elastin components. (A) Fractions 32–35 from Figure 1B. Similar results were obtained when the second peak in Figure 1A (fractions 32–35) or the first peak in Figure 1C (fractions 32–35) was examined under the same conditions. The sample contained about 200 000 cpm and the film was exposed for 1 h. (B) Elastin component isolated from aortas of copper-deficient chicks (Rucker et al., 1975a). The relative absorbance of the autoradiograph (A) and the stained gel (B) was measured as described in Materials and Methods.

Preliminary experiments established that addition of colchicine did not change the nature of the ^{14}C-labeled protein extracted from the cells (see below) but it increased the total amount of intracellular ^{14}C-labeled protein by a factor of about two.

After the incubation, the cells were homogenized in 5 mL of a "standard homogenizing buffer" containing a mixture of protease inhibitors, penicillamine, β-APN, and Nonidet P40. To solubilize the ^{14}C-labeled protein with $NaDodSO_4$, the cell homogenate was centrifuged at 20 000g for 20 min. The pellet was found to contain only 7% of the total ^{14}C-labeled protein and essentially none of the ^{14}C-labeled protein in the pellet was recovered as an elastin monomer after digestion with cyanogen bromide (see below). Therefore, the pellet was discarded. The supernate was lyophilized and the residue was extracted at 100 °C for 5 min with 3.6 mL of 5% $NaDodSO_4$ in 0.01 M sodium phosphate, pH 7.4 (see Materials and Methods). The sample was dialyzed at room temperature against 1% $NaDodSO_4$ in 0.01 M sodium phosphate buffer and examined by gel filtration in $NaDodSO_4$. About 90% of the nondialyzable ^{14}C in the initial cell homogenate was recovered from the column. As indicated in Figure 1A, a large part of the ^{14}C eluted in two peaks. The first peak eluted in the void volume when examined without reduction, but after reduction with 2-mercaptoethanol it eluted on the same position as pro-α chain procollagen (not shown). Therefore the first peak (Figure 1A) appeared to be disulfide-linked pro-α chain procollagen (see Uitto et al., 1976). the second major peak eluted in the same position as the elastin component previously identified in the medium of the same cell system (Uitto et al., 1976). The ^{14}C-labeled protein in the second peak accounted for about 26% of the nondialyzable ^{14}C in the cell homogenate.

Even though protease inhibitors were present in the standard homogenizing buffer, it was possible that some degradation of the elastin component may have occurred during homogenization or subsequent extraction of the intracellular ^{14}C-labeled protein with $NaDodSO_4$ under the conditions employed for the experiment presented in Figure 1A. Therefore, a separate experiment was carried out in which the cell pellet was rapidly dissolved at 100 °C in a buffer solution containing both 5% $NaDodSO_4$ and protease inhibitors (see Materials and Methods), and the sample was then examined in the same gel filtration column equilibrated and eluted with buffer containing both 1% $NaDodSO_4$ and protease inhibitors. The elution profile (not shown) was essentially the same as in Figure 1A, and the elastin component accounted for about 27% of the nondialyzable ^{14}C in the cell homogenate.

To identify further the second peak of ^{14}C-labeled protein shown in Figure 1A, the cell homogenate was also extracted with propanol–butanol under conditions similar to those which have been used to purify "tropoelastin" (Sandberg et al., 1975). The propanol–butanol extract was then examined by gel filtration in $NaDodSO_4$ (Figure 1B). Essentially all the ^{14}C-labeled protein eluted in a single peak with the same elution position as the second peak in Figure 1A. The ^{14}C-labeled protein in the propanol–butanol extract (Figure 1B) accounted for about 17% of the nondialyzable ^{14}C in the initial cell homogenate.

To provide further evidence as to the identity of the second peak in Figure 1A, the cell homogenate was digested with CNBr, a procedure which does not cleave elastin because the protein does not contain methionine (Foster et al., 1975). For CNBr digestion, the cells were homogenized in the standard homogenizing buffer and the homogenate was precipitated with 45% saturation (277 mg/mL) of ammonium sulfate. The ammonium sulfate precipitate was dissolved in 88% formic acid and digested with CNBr (see Materials and Methods). The digest was evaporated to dryness and then examined by gel filtration in $NaDodSO_4$. As indicated in Figure 1C, a peak of ^{14}C-labeled protein was obtained which had the same elution position as the second peak in Figure 1A. As expected, a con-

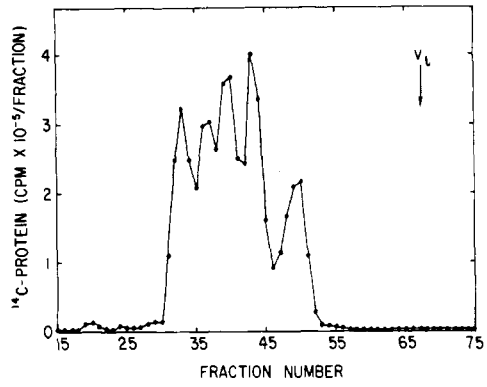

FIGURE 3: Gel filtration in $NaDodSO_4$ of a propanol-butanol extract of matrix-free cells which were frozen for several days before homogenization. The cell homogenate was extracted with propanol-butanol and treated as in Figure 1B. The sample applied to the column corresponded to 1.2×10^9 cells incubated 90 μCi of [^{14}C]proline for 3 h. Chromatographic conditions were the same as in Figure 1.

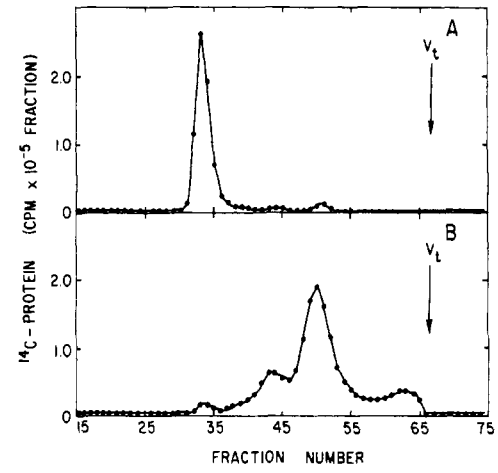

FIGURE 4: Gel filtration in $NaDodSO_4$ of the elastin component extracted from aortas. Aortas and associated blood vessels from ten chick embryos were incubated with 5 μCi of [^{14}C]proline and 65 μg/mL of β-APN for 2 h. The tissues were homogenized and the homogenates extracted with propanol-butanol as described in text. (A) Propanol-butanol extract from aortas and associated large blood vessels. (B) CNBr digest of ^{14}C-labeled protein which was not recovered in the propanol-butanol extract. The pellet obtained by centrifuging the tissue homogenate at 20 000g for 20 min was combined with the pellet obtained after addition of propanol-butanol to the tissue homogenate. The combined samples were digested with CNBr and then extracted with $NaDodSO_4$ (see Materials and Methods). Chromatographic conditions were the same as in Figure 1.

siderable amount of smaller ^{14}C-labeled peptides was also present in the CNBr digest. The elastin component isolated after CNBr digestion accounted for about 21% of the nondialyzable ^{14}C in the initial cell homogenate.

Approximate Size of the Intracellular Elastin Component and of the Elastin Component Secreted into the Medium. The size of the elastin component identified in the cell homogenate was estimated by electrophoresis in $NaDodSO_4$. The peaks of the elastin component isolated by gel filtration on $NaDodSO_4$ (fractions 32 to 35 in Figures 1A–C) were concentrated by evaporation with a stream of N_2 and then examined on 10% polyacrylamide slab gels. The ^{14}C-labeled protein in each of the three samples migrated as a single band (Figure 2A) and standardization of the electrophoretic gels (Weber et al., 1972) with several proteins of known molecular weight indicated that the molecular weight of the elastin component was about 72 000. For comparison, elastin extracted from the aortas of copper-deficient chickens (Rucker et al., 1975a) was also examined in the same system. The soluble elastin from copper-deficient aortas migrated slightly ahead of the intracellular component isolated here, but the difference was close to the limits of resolution of the technique (Figure 2B).

In parallel experiments an estimate was made of the size of the elastin component secreted by the matrix-free cells. As reported previously (Rosenbloom, 1976; Uitto et al., 1976), both procollagen polypeptides and an elastin component were secreted into the medium when the cells were incubated under control conditions but only the elastin component was recovered in the medium if the cells were incubated with 0.3 mM α,α'-dipyridyl. In the experiments carried out here, 1.9×10^8 cells were incubated with 0.3 mM α,α'-dipyridyl and 20 μCi of [^{14}C]proline for 3 h. The medium was separated from the cells by centrifugation and protease inhibitors (Olsen et al., 1976) were added to the medium. Medium proteins were then precipitated with 277 mg/mL of ammonium sulfate and the precipitate was examined by PAGE-$NaDodSO_4$. The major band of ^{14}C-labeled protein (not shown) had essentially the same size as the elastin component isolated from cells. The major band of ^{14}C-labeled protein from the medium appeared less homogeneous and had slightly greater mobility than the intracellular elastin component. The relative migration, however, was clearly less than that of bovine serum albumin (68 000 daltons), and it was indistinguishable from elastin isolated from copper-deficient chick embryos (Figure 2B).

Nonspecific Proteolysis of the Intracellular Elastin Component. In the course of developing the data presented above, several experiments were carried out in which the matrix-free cells were frozen in liquid N_2 at the end of the incubation with [^{14}C]proline. Several days later the frozen pellets were homogenized, without being thawed, in buffer containing protease inhibitors and under the same conditions used for the experiments described above. The homogenates were then treated with propanol-butanol and ^{14}C-labeled protein extracted with propanol-butanol was examined by gel filtration in $naDodSO_4$. As indicated in Figure 3, several peaks of ^{14}C-labeled protein smaller than 72 000 daltons were obtained. Examination by PAGE-$NaDodSO_4$ demonstrated that the approximate size of the ^{14}C-labeled protein in the second peak in Figure 3 was about 55 000 daltons. The results indicated therefore that proteolysis of the elastin occurred if proteases were not appropriately inhibited during processing of the cells.

Extraction of the 72 000-Dalton Component from Aortas of Chick Embryos. As indicated above, most of the elastin component of 72 000 daltons was recovered from the cell homogenates by propanol-butanol extraction (Figure 1B). Therefore the same procedure was tested as a technique for extracting the same component from intact aortas incubated in vitro. Aortas and associated blood vessels from ten 17-day old chick embryos were incubated for 2 h with 5 μCi of [^{14}C]proline. β-APN, 65 μg/mL, was added to inhibit cross-linking of elastin. The tissues were homogenized and the 20 000g supernate from the homogenate was extracted with propanol-butanol. As indicated in Figure 4A, the ^{14}C-labeled protein in the propanol-butanol extract appeared to be the same by gel filtration as the elastin component from the cells (compare Figure 4A with Figure 1B).

To test the effectiveness of the propanol-butanol extraction, all the ^{14}C-labeled protein in the tissue homogenate which was not extractable by propanol-butanol was pooled and digested with CNBr. The CNBr digest was then exhaustively extracted with $NaDodSO_4$. As indicated in Table I, the propanol-bu-

TABLE I: Extraction of [^{14}C]Elastin from Aortas Incubated with β-APN.[a]

Fraction	^{14}C-Protein (cpm × 10^{-5})	(% of total)	[^{14}C]Elastin[b] (cpm × 10^{-5})	(% of total)
P-B extract	7.6	31	6.1	87
CNBr-NaDodSO$_4$ extract	16.8	68	0.51	7
Insoluble residue	0.38	1	0.38	6

[a] Aortas and associated large blood vessels from ten 17-day old chick embryos were incubated for 2 h with 5 μCi of [^{14}C]proline and 65 μg/mL of β-APN. Tissues were homogenized and the 20 000*g* supernate from the homogenate was extracted with propanol-butanol (P-B extract). The two remaining fractions from the homogenate (the 20 000*g* pellet and the precipitate obtained during propanol-butanol extraction) were pooled and digested with CNBr (see text). The CNBr digest was then extracted with NaDodSO$_4$ to give a soluble fraction (CNBr-NaDodSO$_4$ extract) and insoluble fraction (insoluble residue). [b] Gel filtration in NaDodSO$_4$ was used to assay for [^{14}C]elastin in the P-B extract (fractions 32-35 in Figure 4A) and in the CNBr-NaDodSO$_4$ extract (fractions 32-35 in Figure 4B). All the ^{14}C-protein in the insoluble residue was assumed to be cross-linked [^{14}C]elastin. Amino acid analysis of the insoluble residues indicated that it had essentially the same amino acid composition as insoluble elastin from chick aorta (Foster et al., 1975).

TABLE II: Pulse-Chase Experiment with Aortas Incubated under Control Conditions.[a]

Chase Time (min)	Propanol-Butanol Extract (cpm × 10^{-4})	Insoluble Residue (cpm × 10^{-4})
0	13.6	1.0
180	3.4	6.7
300	3.0	9.2

[a] Aortas were pulse labeled and chased as described in Figure 5A and in text. Propanol-butanol extracts and insoluble residues were prepared from the tissues as described in Table I and in text.

tanol extract accounted for 31% of the total ^{14}C-labeled protein in the tissue. The NaDodSO$_4$-soluble ^{14}C-labeled protein recovered after CNBr digestion (CNBr-NaDodSO$_4$ extract) accounted for an additional 68% of the total ^{14}C-labeled protein. Therefore, these two soluble fractions accounted for 99% of the ^{14}C-labeled protein in the tissue. However, only a small amount of the ^{14}C-labeled protein in the CNBr-NaDodSO$_4$ extract was as large as the elastin component extracted from cells (compare Figures 4B and 1C). The results indicated therefore that the propanol-butanol procedure extracted most of the 72 000 component of elastin from the tissue.

A parallel series of experiments were carried out with aortas which were incubated for 3 h with [^{14}C]proline but without an inhibitor of cross-linking. The elution profile of the propanol-butanol extract and the CNBr-NaDodSO$_4$ extract were similar to those shown in Figures 4A and 4B. However, about one-third of the total ^{14}C-labeled protein was in the insoluble residue. The amino acid composition of the insoluble residue (not shown) was similar to that of cross-linked elastin fibers (Foster et al., 1975) and therefore the ^{14}C-labeled protein in the residue probably represents cross-linked [^{14}C]elastin.

Pulse-Chase Experiments with Aortas. Since the propanol-butanol procedure effectively extracted the 72 000-dalton elastin component from aortas, it was used in pulse-chase experiments. Aortas were incubated for 1 h with 3 μCi of [^{14}C]proline and the label was chased by transferring the tissue to fresh medium containing 500 μg/mL of [^{12}C]proline.

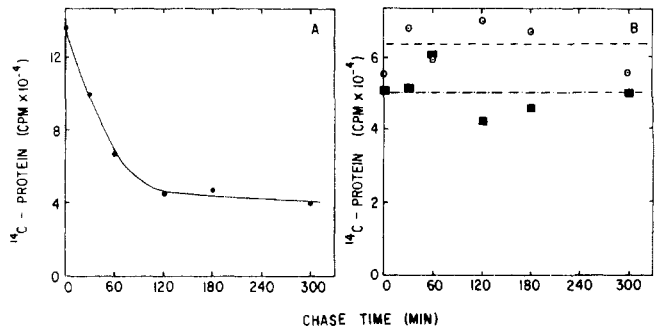

FIGURE 5: Pulse-chase experiments in aortas. Aortas and associated blood vessels were pulse labeled with 3 μCi of [^{14}C]proline for 1 h as described in text and then the label was chased, transferring the samples to fresh medium containing 500 μg/mL of [^{12}C]proline. The medium was lyophilized and the tissue was homogenized before treatment with propanol-butanol as described in Materials and Methods. Values indicate total ^{14}C-labeled protein extracted from both the tissues and the medium with the propanol-butanol procedure. (A) Tissues pulse labeled and chased under control conditions. (B) Tissues pulse labeled and chased in the presence of 1 mg/mL of penicillamine (O - - - O) or in the presence of 65 μg/mL of β-APN (■ - · - · - ■).

When the tissues were incubated without an inhibitor of cross-linking, the amount of ^{14}C-labeled elastin which was extractable in propanol-butanol decreased during the chase period, and after 120 min it was about one-third of the initial value (Figure 5A). Most of the ^{14}C-labeled protein which was lost from this fraction during the chase period appeared in the insoluble residue from the tissue (Table II).

The pulse-chase experiment was also carried out in the presence of either penicillamine or β-APN. With either inhibitor of cross-linking, the total amount of ^{14}C-labeled protein extractable with propanol-butanol (from tissue plus medium) remained constant even with chase periods of 5 h (Figure 5B). The results suggested therefore that the agents effectively blocked cross-linking. They also suggested that the decrease of ^{14}C-labeled protein extractable with propanol-butanol from the control tissue (Figure 5A) represented a conversion of the 72 000-dalton component to insoluble fibers (Table II).

Persistence of the 72 000-Dalton Component in Tissues Incubated with β-APN or Penicillamine. In further studies the size of the ^{14}C-labeled protein in the propanol-butanol extracts of aortas was examined by electrophoresis. As expected from previous observations (Figure 4A and Table I), only the 72 000-dalton component was present when the tissues were extracted at the end of a 1-h labeling period with [^{14}C]-proline (Figures 6A and 6B). In tissues labeled and chased in the absence of an inhibitor, there was a decrease in the amount of 72 000-dalton component (Figure 6A), presumably because it was converted to cross-linked elastin (see above). Even with chase periods of 300 min, however, there was no evidence of elastin components smaller than 72 000 daltons in the propanol-butanol extract. When the tissues were incubated with penicillamine, there was no change in the 72 000-dalton component with chase periods of up to 5 h (Figure 6B).

Similar results were obtained with tissue incubated with β-APN and chased for 2 h. In samples incubated with β-APN and chased for more than 2 h, there was a gradual decrease in the amount of the 72 000-dalton component in the tissue (not

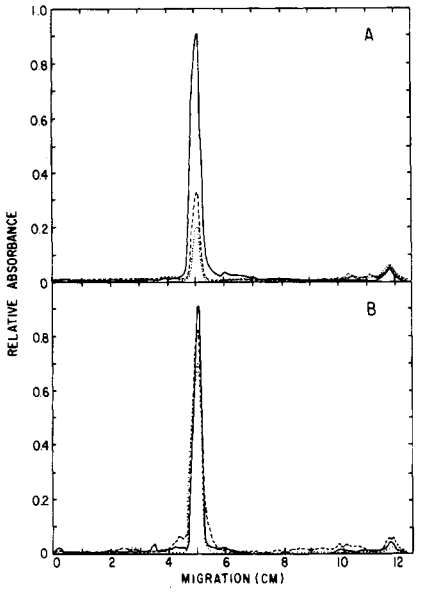

FIGURE 6: Electrophoresis in $NaDodSO_4$ of ^{14}C-labeled protein components extracted with propanol-butanol. Conditions were as in Figure 5. Samples consisted of propanol-butanol extracts of tissues only. The autoradiographs were scanned as indicated in text. (A) Control samples. (B) Sample incubated with 1 mg/mL of penicillamine. Symbols: Chase period of 0 min (—), 120 min (- - -), and 300 min (• • •).

TABLE III: Effects of Penicillamine and β-APN on the Recovery of ^{14}C-Protein Extracted from Aortas with Propanol-Butanol[a]

		^{14}C-Protein Extracted with Propanol-Butanol		
	Chase Time (min)	Tissue (cpm × 10^{-3})	Medium (cpm × 10^{-3})	Fraction of Initial ^{14}C-Protein Recovered in Medium (%)
Control	0	136.3	0	0
	30	95.6	4.5	3.3
	60	60.7	5.8	4.2
	120	38.4	7.1	5.2
	180	34.3	12.9	9.5
	300	29.8[b]	10.3	7.5
Penicillamine	0	55.0	0	0
	30	68.0	0.3	0.5
	60	58.0	1.2	2.2
	120	66.2	3.8	6.9
	180	61.0	6.1	11.1
	300	49.0	4.4	8.0
β-APN	0	50.5	0	0
	30	50.9	0.4	0.8
	60	57.0	3.0	6.0
	120	35.5	6.5	12.9
	180	34.5	11.0	21.8
	300	25.5	24.5	48.5

[a] Aortas were pulse labeled and chased as indicated in Figure 5. Tissue and medium were examined separately for ^{14}C-labeled protein extractable in propanol-butanol. [b] Because elastin component was incorporated into cross-linked fibers, there was relatively rapid decrease in the total ^{14}C-labeled protein extractable with propanol-butanol under control conditions. As indicated in Figure 5B, the total amount of ^{14}C-labeled protein extractable in propanol-butanol (tissue plus medium) did not decrease in the presence of penicillamine or β-APN.

shown). The decrease in the 72 000-dalton component in the presence of β-APN was not, however, accompanied by any increase in small ^{14}C-labeled peptides in the tissue. Instead, it was accounted by a loss into the medium of ^{14}C-labeled protein extractable in propanol-butanol (Table III). A small part of the propanol-butanol extractable ^{14}C-labeled protein in the medium was about the same size as the elastin component isolated from cells, but most was recovered as small peptides. The peptides migrated near the dye front and had apparent molecular weights of less than 25 000.

Discussion

As indicated by several previous reports (Murphy et al., 1972; Rucker et al., 1975b; Schofield and Harwood, 1975; Uitto et al., 1976), the aorta and associated large blood vessels from chick embryos provide a useful system for studying the biosynthesis of elastin in vitro. Also, matrix-free cells prepared from these tissues can be incubated in suspension and can be used to examine secretion of elastin (Schofield and Harwood, 1975; Uitto et al., 1976; Rosenbloom and Cywinski, 1976).

The results presented here demonstrated that if the matrix-free cells are rapidly boiled in buffer containing high concentrations of protease inhibitors and $NaDodSO_4$, a major fraction of the intracellular protein is recovered as an elastin component with an apparent molecular weight of 72 000. Although proteolysis during extraction with these conditions cannot be completely excluded, it seems highly unlikely. The elastin component secreted into the medium by the matrix-free cells also had an apparent molecular weight of about 72 000 daltons. The results, therefore, support previous suggestions (Abraham et al., 1974; Narayanan et al., 1976; Rosenbloom and Cywinski, 1976; Uitto et al., 1976) that elastin is secreted from cells as a polypeptide of about 72 000 daltons.

It should be noted that the results do not exclude the possibility that the first elastin polypeptide synthesized in cells is larger than 72 000 but is cleaved to about 72 000 daltons before the molecule is secreted. Several such precursor proteins, or "pre-proteins", have been identified and it has been shown that the pre-proteins contain NH_2-terminal sequences which are removed soon after the polypeptides enter the cisternae of the rough endoplasmic reticulum (see Milstein et al., 1972; Judah et al., 1973; Blobel and Dobberstein, 1975). If a pre-protein form of elastin exists, it would have escaped detection here if it had a rapid metabolic turnover and therefore constituted only a small fraction of the total intracellular ^{14}C-labeled protein.

To follow the fate of the 72 000 component, a series of pulse-chase experiments were carried out with isolated aortas. Studies on elastin biosynthesis in vitro and in vivo are in part hampered by the limited solubility of the protein either in the monomer form or in the highly cross-linked form found in fibers. However, treatment of tissue homogenates with a minor modification of the propanol-butanol procedure of Sandberg et al. (1975) made it possible to isolate most of the newly synthesized elastin with an apparent molecular weight of 72 000. In pulse-chase experiments under control conditions, about two-thirds of the 72 000-dalton component disappeared from the propanol-butanol extract in 2 h. Since β-APN and penicillamine inhibited the disappearance, the loss of ^{14}C-labeled protein from the propanol-butanol extract apparently represented incorporation of the 72 000-dalton component into cross-linked fibers. A similar, rapid conversion of "soluble" elastin in aortas was recently reported by Narayanan and Page (1976). In the experiments reported on here, a small fraction of the elastin remained in a form extractable with propanol-butanol and the fate of this fraction is unclear.

Examination of propanol-butanol extracts from the pulse-

chase experiments did not reveal any cleavage of the 72 000-dalton component. In tissues incubated without an inhibitor of cross-linking, there was no evidence of smaller ^{14}C-labeled polypeptides in the propanol-butanol extracts even with chase periods of up to 5 h. It seems reasonable to assume that smaller elastin components would have been extracted from the tissue by propanol-butanol under the conditions employed here (see Figure 3 above and Sandberg et al., 1975). When the pulse-chase experiments were carried in the presence of penicillamine, the 72 000-dalton component remained in the tissue for up to 5 h without any apparent proteolysis of the molecule. In the presence of β-APN, the component remained intact for 2 h and thereafter a small amount was recovered as small ^{14}C-labeled peptides in the incubation medium. The data therefore speak against the hypothesis that newly secreted elastin is cleaved to an intermediate of considerably smaller size before it is converted into cross-linked fibers.

The failure to find a smaller intermediate does not completely exclude the possibility of such an intermediate, but formation of a smaller intermediate can be reconciled with the observations made here only if several unlikely assumptions are made. In the case of aortas incubated under control conditions, the data are consistent with a smaller intermediate only if it is assumed that the intermediate represents only a small fraction of the newly synthesized elastin in the tissue or that it is not extractable with the propanol-butanol procedure employed here. In the case of aortas incubated with penicillamine, it would be necessary to assume that, in addition to inhibiting cross-linking, penicillamine inhibited proteolytic cleavage of the 72 000-dalton monomer so that no smaller elastin component was seen even after prolonged chase periods. A similar assumption would be necessary to explain the data obtained by incubating the tissues with β-APN for 2 or 3 h.

The major evidence for an elastin monomer of about 57 000 daltons comes from experiments in vivo in which cross-linking was inhibited with β-APN (Sykes and Partridge, 1974; Foster et al., 1975). As observed here, however, incubation of aortas with β-APN for more than 2 or 3 h appears to promote proteolysis which is not seen either under control conditions or in the presence of penicillamine. The cleaved elastin components recovered in the medium of tissues incubated with β-APN were less than 25 000 daltons, and they are unlikely to be specific precursors of elastin fibers.

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