Additional Evidence for a Proform to Tropoelastin from Chick Aorta

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Evidence is presented for the presence of a precursor to tropoelastin in chick arterial extracts. The precursor is approx. 100000 daltons in size. It is suggested to be a precursor to tropoelastin (72000 daltons). This protein may be observed in culture *in vitro* if appropriate precautions are taken to inhibit proteolysis. Once synthesized, it appears to be converted into tropoelastin within 10–20 min. The protein may also be detected *in vivo*. When 1-day-old cockerels were fed on a copper-deficient diet (<1 p.p.m. to inhibit cross-linking) containing ϵ -aminohexanoic acid (0.2%) to retard proteolysis and then injected with [3 H]valine, extraction of arterial proteins 12 h after injection resulted in detection of two major peaks of [3 H]valine-labelled protein with pI values of pH 7.0 and 5.0 respectively. The protein that focused at pH 7.0 was estimated to be about 100000 daltons in size and could be shown to be converted into a more basic protein with the properties of tropoelastin. It is speculated that the protein with pI 5.0 may be yet another extension peptide. The data appear to be in keeping with similar observations by ourselves and others that a proform of tropoelastin exists, and, in at least one step before conversion into tropoelastin, exists as a 100000-dalton protein subunit.

Elastin is the primary elastomer in tissues that demonstrate compliance. It is present in large arterial vessels in amounts that range from 20 to 50% of the total protein. It is also a major constituent in elastic ligaments and lung. Morphologically distinct filaments of elastin are also found in skin and other connective tissues (Sandberg, 1976; Rucker & Tinker, 1977).

In arterial tissue, the smooth-muscle cell appears to be responsible for the synthesis of elastin (Ross, 1973). Elastin subunits are secreted from smooth-muscle cells along with microfibrillar components that appear to be involved in the initial registration and alignment of the elastin subunits. Once appropriate alignment is made, the soluble elastin subunits are cross-linked via mechanisms involving the enzyme lysyl oxidase (Rucker & Murray, 1978), and the microfibrillar components are then removed (Ross, 1973). In its mature state the elastic fibre is extremely insoluble in physiological solutions due to cross-linking. Further, once the microfibrillar components are removed, the elastic fibre becomes more apolar because of the high proportions of neutral and apolar amino acid residues in elastin (Sandberg, 1976; Rucker & Tinker, 1977).

One of the non-cross-linked precursors to insoluble elastin is often referred to as tropoelastin. Tropoelastin is approx. 72000 daltons in size. We have previously reported on its isolation and partial characterization from the aortas of copper-deficient

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

chicks (Rucker *et al.*, 1975a). The key to the isolation of soluble elastins is the inhibition of lysyl oxidase, which in arterial tissue is copper-dependent. Others have isolated the protein from lathyrotic chicks (Sykes & Partridge, 1974; Foster *et al.*, 1975), copper-deficient pigs (Smith *et al.*, 1972; Sandberg, 1976) and copper-deficient calves (Whiting *et al.*, 1974). Various features pertaining to these isolations and the characterization of tropoelastin may be found in recent reviews (Sandberg, 1976; Rucker & Tinker, 1977).

The subject of the present paper relates to the existence of non-cross-linked elastins other than tropoelastin. Chick aortas in culture and *in vivo* appear to synthesize an elastin-like subunit that is approx. 100000 daltons in size. This protein appears to be a proform of tropoelastin that is rapidly metabolized to tropoelastin. The data represent an extension of our previous work on the detection of a proform to tropoelastin (Rucker *et al.*, 1977) and work by Foster *et al.* (1978).

Experimental

Preparation of tropoelastin and diets

Tropoelastin was isolated from the aortas of copper-deficient chicks and was used as an electrophoretic marker in several experiments. To prepare this form of soluble elastin, 1-day-old chicks were fed on a copper-deficient diet (<1 p.p.m.) plus 400 p.p.m. of zinc as a copper antagonist (Rucker & Riemann, 1972; Tom *et al.*, 1977). At 18 days the birds were

killed and the aortas quickly excised and processed for the isolation of tropoelastin by the procedure of Sandberg *et al.* (1975) as modified by Rucker *et al.* (1975a). In addition, 1-day-old chicks were also fed on the same diet, but with 0.2% ϵ -aminohexanoic acid added in an attempt to retard the proteolysis of soluble elastins *in vivo*. Aortas from these chicks were used to establish the presence of putative proforms to tropoelastin (cf. studies *in vivo*). In addition, aortas from 3-week-old chicks fed on a copper-supplemented diet (25 p.p.m. of Cu) were used when tissue-culture techniques were used (Rucker & Riemann, 1972). The anterior thoracic aorta was used for the cultures. Other details pertaining to care and handling of the animals have already been published (Rucker & Riemann, 1972).

Radiochemical-labelling experiments in vitro

For the experiments *in vitro*, the aortas were quickly removed, cleansed of adhering adipose tissue and minced (1 mm³ segments) in Medium 199 containing Hanks' salts, glutamine at 25 mM, and Hepes as buffer (Grand Island Biological Company, Grand Island, NY, U.S.A.). The minced tissue was then transferred to incubation flasks containing the same medium, but with 50 μ Ci of L-[2,3-³H]valine/ml added (sp. radioactivity 30 Ci/mol; Amersham/Searle Corp., Des Plaines, IL, U.S.A.). The flasks were oxygenated with a mixture of O₂/CO₂ (19:1, v/v). The tissue (1 g wet wt./9 ml of medium) was then incubated at 41°C for varying times in the manner of a pulse-chase experiment. For example, the tissue was either 'pulsed' for 15, 30, 60 or 120 min in the medium containing [³H]valine. After the 15 or 30 min incubations, however, portions of the tissue were removed and transferred to Medium 199 containing no [³H]valine and five times the initial L-valine concentration (3 mM) plus 15 μ g of cycloheximide/ml. Under these conditions the [³H]valine-labelled products were 'chased' for an additional 15 or 30 min. After each time period, approx. 0.5 g of tissue was removed from the medium. The tissue was quickly washed over a nylon-mesh screen with chilled water by means of a suction flask. The tissue was then homogenized directly into a 10 ml solution containing 1% ampholyte (Bio-Lyte 3/10; Bio-Rad Laboratories, Richmond, CA, U.S.A.) and 25 μ g of human α_1 -antitrypsin (Worthington Biochemicals, Freehold, NJ, U.S.A.) as a proteolytic inhibitor (cf. Mecham & Foster, 1977). After the homogenization, the tissue was centrifuged at 25000g for 30 min. The layer of floating lipid was removed and the supernatant fraction was then used as a source of radioactively labelled protein for characterization. In each case, the total time from homogenization to the initiation of steps for characterization involved no more than 60 min.

Polyacrylamide-gel electrophoresis

³H-labelled proteins extracted from the tissue were partially characterized by polyacrylamide-gel electrophoresis techniques by using 5% (w/v) acrylamide gels containing 0.1% SDS or by isoelectric focusing in 7.5% polyacrylamide gels containing ampholytes. For electrophoresis in gels containing SDS, the method of Furthmayer & Timpl (1971) was used. Portions of the supernatant fraction containing 200–300 μ g of protein (100 μ l) were first mixed with an equal volume of a solution containing 8 M-urea, SDS (1%, w/v) and 2-mercaptoethanol (2%, v/v). A portion (100 μ l) of this mixture was then applied to the gels. After electrophoresis the gels were sliced into contiguous 2 mm segments. Individual segments were then digested in 10 ml of a Protosol/Econofluor (3:97) mixture (New England Nuclear Corp., Boston, MA, U.S.A.) for 12 h (30°C) and counted for radioactivity with a Searle mark I liquid-scintillation counter (counting efficiency for ³H, approx. 45%). Duplicate gels were run for each sample. Gels that were not sliced were fixed (25%, w/v, trichloroacetic acid) and stained with Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 4–6 h. The gels were destained with a mixture of methanol/acetic acid/water (5:1:6, by vol.). The mobilities of bovine serum albumin, tropoelastin and ovalbumin (Sigma) were used for estimates of molecular size. 2-Mercaptoethanol was not added to the protein standard mixtures, however, so that the mobility of the albumin dimer and trimer could be estimated.

For isoelectric focusing, portions of the supernatant fraction were electrophoresed on 7.5% polyacrylamide gels containing a 1% mixture of Bio-Lyte ampholytes. The gels were prepared as described by Furthmayer & Timpl (1971), but the phosphate buffer and SDS were replaced by a 1% ampholyte mixture. For the mixture, ampholyte solutions ranging from pH 7–10 and pH 3–10 were mixed at a ratio of 3:2. L-Arginine and L-lysine were also added (0.06%) to stabilize the cathode end of the gels. The gels were approx. 8 cm long. The samples were applied in a 40% solution (100 μ l) of sucrose. A 10% (w/v) sucrose solution (300–500 μ l) was laid over the sample layer. A 0.5 M-NaOH solution was used as the cathode solution. H₂SO₄ (0.5 M) was used as the anode solution. An initial current of 0.5 mA/gel and 100 V were applied. After 18 h, the voltage was increased to 800 V. The gels were focused after 20 h. The mobility of tropoelastin, cytochrome c, Bromophenol Blue and bovine serum albumin, in addition to direct estimates, were used to determine the pH gradient of the gels. The distribution of radioactivity was again established by slicing the gels in 2 mm contiguous sections followed by liquid-scintillation counting as described above.

Studies in vivo

To investigate whether the observations obtained *in vitro* could be duplicated *in vivo*, an experiment was carried out with the chicks fed on the copper-deficient diet containing 0.2% ϵ -aminohexanoic acid for 17 days. Before tissue preparation, each bird (50 in total) was injected with 60 μ Ci of [3 H]valine/100g body wt., 12h before being killed by cervical dislocation. In this case, their aortas were removed, minced and homogenized into a solution of 1% ampholyte (pH3–10) containing 50 μ g of α_1 -anti-trypsin/ml (tissue wt./v, 1:9). The homogenate was centrifuged at 25000g for 30min. The supernatant fraction was then subjected to isoelectrofocusing on a flat bed of Sephadex G-75 (Superfine grade; Pharmacia, Uppsala, Sweden) containing 1% ampholyte (pH3–10) and 0.06% L-arginine and L-lysine. The technique for focusing has been described elsewhere by Radola (1969). After the focusing step, the slab was cut into 1 cm segments and samples of the gel were eluted with 0.5M-acetic acid (cathode end to the region of pH7.5 in the slab) or 0.1M-pyridine (anode end to the region of pH7.5 in the slab). Radioactivity in portions of these fractions was then estimated. The fractions containing radioactivity were then concentrated by freeze-drying and a portion (equivalent to 10^5 – 10^6 d.p.m.) was dissolved in solutions appropriate for either refocusing or electrophoresis in the presence of SDS as described above. The mobility of the labelled protein subunits in the gels was determined after electrophoresis.

In one experiment, portions of gels (4mm segments) after isoelectric focusing that corresponded to labelled peaks of protein were also electrophoresed in gels containing SDS. This was accomplished by taking the gel slice after isoelectric focusing and pulverizing it in a small volume (100–200 μ l) of 0.1M-sodium phosphate buffer, pH7.6, containing SDS (1%), 2-mercaptoethanol (2%) and urea (8M). As much of this material as possible (approx. 100 μ l) was layered on polyacrylamide gels containing SDS and electrophoresed. This was carried out in an attempt to size protein for which the isoelectric point had been estimated.

Furthermore an attempt was made to partially characterize the protein fraction that focused at pH7.0, since it appeared to contain the tropoelastin precursor. After concentration (by freeze-drying) this fraction was suspended in a solution containing 0.05M-sodium phosphate buffer, pH7.6, plus 0.1% SDS. The fraction (approx. 6mg of protein) was then applied on a column (1.5cm $\times$ 50cm) of Sephacryl S-200 (Pharmacia) and eluted with the same solution. Fractions of 3ml volume were collected. Radioactivity and protein were determined. The fractions that were eluted with an apparent mobility corresponding to 100000 daltons from the Sephacryl column

were collected and then dialysed against increasing concentrations of methanol (e.g. 10, 20, 40 or 80%, v/v). This was done in an attempt to remove the SDS. At this point the methanol was removed under vacuum and the protein residue was resuspended in 0.5M-acetic acid to which 2vol. of n-propanol was added slowly at 4°C. This step was followed by the addition of 5vol. of n-butanol. The mixture was then filtered. Protein soluble in the aqueous alcohol mixture was evaporated and again taken to dryness. The rationale for the alcohol-fractionation step was based on tropoelastin-isolation procedures outlined by Sandberg *et al.* (1975). After alcohol fractionation, the protein was fractionated further by first dissolving the protein into phosphate buffer containing proteinase inhibitors (Foster *et al.*, 1975), and then slow addition of NaCl to 15% (w/v). The protein that was precipitated by NaCl was recovered (0.5mg) by centrifugation and was dialysed against a solution containing 0.05M-phenylmethanesulphonyl fluoride and freeze-dried in preparation for isoelectric focusing to assess purity.

Results*Evidence for a proform to tropoelastin*

Some results from studies *in vitro* on the detection of a proform to tropoelastin are given in Figs. 1 and 2. The data were obtained from the pulse-chase experiments in which an attempt was made to radiochemically label precursors to tropoelastin and show their conversion into tropoelastin. The results in Fig. 1 represent the distribution of radioactivity in protein subunits extracted from aortas after a 15 min pulse in medium containing [3 H]valine followed either by a 15 min chase or a 30 min chase in medium containing no radioactive isotope, but cycloheximide. The protein extracts were applied either to polyacrylamide gels containing SDS or ampholyte.

The distribution of radioactivity after the 15 min pulse indicates a major peak of [3 H]valine-labelled protein migrating to a position corresponding to 100000 daltons. As shown, this component appears to be cleaved very rapidly, after the 15 or 30 min chase, to a protein subunit with the mobility of tropoelastin and smaller-molecular-weight peptides. Coincident with the apparent cleavage is also a shift in the electrophoretic mobility of the material in isoelectric-focusing gels. The 100000-dalton component initially focused near pH7.0. This represents a pI substantially lower than might be expected of tropoelastin, a relatively basic protein. Concomitant with the shift in molecular size from approximately 100000 to 70000 daltons, the protein that focused at pH7.0 appeared to be converted into a protein subunit with the pI of tropoelastin (near pH8.5). The 'open' peaks in the Figures correspond to the pI of [3 H]valine.

Fig. 1. Distribution of radioactivity from chick arterial extracts after electrophoresis in 1% SDS/polyacrylamide gels (left) or by isoelectric focusing (right)

Minced chick aorta was first 'pulsed' in Medium 199 containing [^3H]valine for 15 min. A portion of the tissue was then homogenized and the resulting solubilized proteins were partially characterized as described in the text, or a portion of the labelled tissue was transferred to 'chase' medium and incubated for an additional 15 or 30 min before homogenization and partial characterization. For the gels containing SDS (a_{SDS} , b_{SDS} , c_{SDS}), the arrow to the left represents the mobility of protein of approx. 100000 daltons in size. The arrow to the right represents protein with the mobility of tropoelastin. In isoelectric-focusing gels, the major radioactively labelled protein product appears at pH 7.0 during the pulse (a_{IF}). During the chase periods (15 min, b_{IF} ; 30 min, c_{IF}), this product appears to be converted into a product with a pI of about 8.5. The 'open' peak in the Figures corresponds to [^3H]valine. [^3H]Valine was removed from the gels containing SDS during fixation (see the text).

The isoelectric-focusing gels were not fixed in 25% trichloroacetic acid as was the case for the gels containing SDS. Subsequently [^3H]valine and other small-molecular-weight peptides were not removed from the isoelectric-focusing gels.

With a 30 min pulse (results not shown), more of the 100000-mol.wt. component was converted into lower-molecular-weight protein products relative to the distribution of protein subunits after the 15 min pulse. In the isoelectric-focusing gels several radio-

actively labelled bands of protein were observed near pH 7.0 and 8.5. During the subsequent chases (15 or 30 min), there was again the almost complete disappearance of the labelled protein peak corresponding to a pI of pH 7.0 and approx. 100000 daltons with the emergence of a well-defined protein subunit possessing the properties of tropoelastin in the two gel systems.

In contrast with the results given in Fig. 1, the 100000-dalton component was not significantly observed relative to radioactivity corresponding to tropoelastin, if the initial incubation in the presence of [^3H]valine was 60 min or longer (Fig. 2). It seems

that most of the 100000-dalton component is cleaved within 15 to 30 min of the initial incorporation of [^3H]valine. Furthermore it was observed that when α_1 -antitrypsin was omitted from the extraction medium, only a tropoelastin-like component was observed, regardless of the incubation time. This was also the case when organic acids, such as 0.5 M-acetic acid or formic acid, are used as extractants (cf. Rucker *et al.*, 1977).

With respect to the estimates of molecular size in these experiments, the values, although not arbitrary, are certainly not precise. The size of the radioactively labelled protein subunits are at best approximations.

Fig. 2. Distribution of radioactivity in chick arterial extracts after a 60 min (a_{SDS} , a_{IF}) or 120 min (b_{SDS} , b_{IF}) incubation in Medium 199 containing [^3H]valine

Most of the radioactivity migrates as tropoelastin in gels containing SDS (a_{SDS} , b_{SDS}) or ampholyte (a_{IF} , b_{IF}). Relative to the peaks corresponding to tropoelastin, little of the putative proform of tropoelastin is observed. For further details see the legend to Fig. 1.

It is well recognized that collagen α -chains are retarded in polyacrylamide gels containing SDS and that the carbohydrate content of protein may influence electrophoretic mobility (Furthmayer & Timpl, 1971). In 5 or 7.5% polyacrylamide gels containing SDS, our tropoelastin standard migrates slightly above bovine albumin monomer. The putative tropoelastin precursor migrated with a mobility between that of the bovine albumin monomer and dimer, and was slightly retarded with respect to the mobility of the ovalbumin dimer.

Evidence for a proform to tropoelastin from studies in vivo

The results shown in Fig. 3 represent the mobility of radioactivity in a chick arterial extract when focused on a slab of Sephadex G-75 (superfine grade). In previous studies (Rucker & Riemann, 1972; Rucker *et al.*, 1975a; Tom *et al.*, 1977), we have observed that 10–20mg of protein may be extracted

per g of fresh aorta from copper-deficient chicks. About 20–30% of this protein may be isolated as tropoelastin (Rucker *et al.*, 1975a). Also, in the 3-week-old copper-deficient chick soluble elastin appears easily labelled *in vivo* by using [^3H]valine as a marker relative to its incorporation into other proteins (Rucker *et al.*, 1975b). It is noteworthy that immediate isoelectric focusing of the arterial extracts on Sephadex gel indicated little material with properties of tropoelastin, i.e. the presence of radioactively labelled protein with a pI greater than pH 8.0. Two major radioactive peaks of protein were observed in the Sephadex slab. Peak A focused at pH 7.0. Peak C focused at approx. pH 5.0.

However, when the material was refocused 18h later, after freeze-drying, peak A was modified (Figs. 3 and 4). Three major peaks of protein were observed as shown in the insert of Fig. 3. The majority of the radioactivity migrated as a 100000-dalton component, but in addition, proteins with the mobility of tropoelastin and a 30000-dalton protein subunit were observed. The majority of the radioactivity in peak C (inset, Fig. 3), appeared unmodified with a mobility corresponding to approx. 45000 daltons. With respect to the protein components eluted as peak A, the results from the SDS/polyacrylamide-gel electrophoresis and isoelectric focusing suggested that this

Fig. 3. Distribution of radioactivity after isoelectric focusing of an arterial extraction from copper-deficient chicks on a slab of Sephadex G-75 (superfine grade)

The peaks designated A and C correspond to pI values of pH 7.0 and approx. pH 5.0 respectively. Note that there is little evidence for the presence of tropoelastin (pI near or greater than pH 8.5) in the extract. To label the proteins in the extract, chicks were injected with [^3H]valine 12h before extraction and focusing. Both peaks A and C were recovered from the slab and a portion was re-electrophoresed on SDS/polyacrylamide gels. —, Radioactivity; ----, pH. The inset shows the distribution of radioactivity on 5% SDS gels. The solid line corresponds to products from peak A and the mobilities of the three major peaks represent proteins with mobilities of 100000, 70000 and 30000 daltons respectively. Almost all of the material in peak C (----) corresponded to protein with a mobility of 45000 daltons (cf. Fig. 4).

Fig. 4. Isoelectric focusing on polyacrylamide gels containing ampholyte of the material eluting as peaks A or C given in Fig. 3

Portions of protein from peaks A (—) or C (----) were applied to the isoelectric-focusing gels. Peak C focused at pH 5 and appeared unaltered during the time required to prepare the samples. Peak A focused as two components on the isoelectric-focusing gels with pI values at pH 7.0 and near that of tropoelastin (pH 8.5). ----, pH.

was due to cleavage (presumably 100000 daltons $\rightarrow$ 70000 daltons + 30000 daltons). For example, in common with the observations from the studies *in vitro*, there was a shift in the pI of some of the material from pH 7.0 to approx. pH 8.5 (Fig. 4). Subsequently these data were taken as an additional indication that the 100000-dalton component is converted into a protein subunit with the properties of tropoelastin.

When slices from the isoelectric-focusing (peak C) gels were taken and re-electrophoresed on SDS/polyacrylamide gels, a band of protein was observed after staining. Its mobility corresponded to a protein with an apparent molecular size of 45000 daltons. When material corresponding to peak A was re-focused and electrophoresed on SDS/polyacrylamide gels in a similar manner, it was observed that the protein that focused at pH 7.0 in the isoelectric-focusing gels as radioactivity appeared to migrate in SDS/polyacrylamide gels as two components with mobilities corresponding to 30000 and 100000 daltons. The protein that focused at pH 8.5 appeared to migrate with the mobility of tropoelastin. The gels are not shown because the respective bands of protein did not stain with an intensity suitable for photography.

In common with the results shown in Fig. 3, chromatography of peak A with a column of Sephacryl S-200 resulted in peaks of radioactively labelled protein with elution characteristics of molecules of approx. 100000 and 30000 daltons (Fig. 5). The 'shoulder' after the first peak corresponds to the mobility of tropoelastin (Rucker *et al.*, 1978). Furthermore, when attempts were made to partially purify the leading peak of protein from the Sephacryl column, it was observed that this material had the characteristics of a soluble elastin. For example, over 90% of the radioactivity corresponding to the peak remained soluble when dialysed against increasing concentrations of methanol to remove SDS. When evaporated to near dryness and suspended in 0.5M-acetic acid, the radioactivity could be solubilized on addition of n-propanol and n-butanol (85% of the total radioactivity was recovered with this step). Also, the radioactivity could be solubilized into 0.05M-sodium phosphate buffer (pH 7.6, 4°C) after the removal of n-propanol and n-butanol by evaporation under vacuum. The radioactivity could then be precipitated by the slow addition of NaCl to 15% (w/v). Approx. 60% of the original starting material as radioactivity was recovered in the precipitate.

When the precipitate was isoelectric-focused on polyacrylamide gels, a sharp band of the protein was observed at pH 7 (Fig. 6). Also shown is a gel containing purified tropoelastin. Other material is seen at the cathode end of the gel (presumably this material could not penetrate the gels). In addition, some material appeared to focus near the pI of cytochrome c (pH 10.4). Interestingly, tropoelastin focused at

Fig. 5. Elution pattern from a column of Sephacryl S-200 of peak A obtained after isoelectric focusing (Fig. 3). Protein was determined with Coomassie Brilliant Blue G-250 (Bio-Rad protein-determination kit). An absorbance (—) of 0.5 is equivalent to approx. 50 μ g of serum protein. The radioactivity counts in the first peak are retarded with respect to the void volume (fraction 10). The three peaks of radioactivity (----) correspond to approx. 100000, 70000–80000, or 30000–35000 daltons respectively. The column (1.5cm $\times$ 60cm) was run under pressure at 60ml/h. Other details are given in the text.

pH 8.5. Although this is in keeping with results shown in the previous Figures, one would assume, on the basis of its amino acid composition, a more basic pI. Others have also observed apparent anomalies related to the mobility of tropoelastin in isoelectric-focusing gels (Roensch *et al.*, 1972).

Discussion

The present work demonstrates that chick aorta is capable of synthesizing a protein that possesses properties befitting a precursor to tropoelastin. At least one form of the protein has an apparent molecular size of about 100000 daltons. Its conversion into tropoelastin appears to occur within minutes in cultures *in vitro* and is only detected when high concentrations of appropriate proteinase inhibitors are present throughout the extraction steps. This observation is also consistent with the observations recently reported by Foster *et al.* (1978), who have shown that rabbit smooth-muscle cells in culture synthesize a proform of elastin (120000–140000 daltons) that appears to be metabolized to tropoelastin through a 90000–100000-dalton intermediate. Although under the conditions we have used, the high-molecular-weight species was not observed, it may be speculated that the apparent 100000-dalton protein on combination with the apparent 45000-dalton component could provide an initial product with the appropriate

Fig. 6. Isoelectric focusing of tropoelastin and peak A (Fig. 3) after gel filtration, alcohol fractionation and NaCl fractionation

The gels contained 1% ampholyte (pH3–10) plus L-arginine and L-lysine to stabilize the cathode end (top). Gel (a) is tropoelastin prepared as described by Rucker *et al.* (1975a). It was used as one of the reference proteins and marker in the characterization of the radioactively labelled components. This material yielded a single band when electrophoresed on polyacrylamide gels containing SDS (results not

molecular size. Obviously the nature of the cleavage steps and the importance of the cleavage products to elastogenesis still requires considerable clarification.

The key to observing a putative proform to tropoelastin in culture is the use of appropriate inhibitors throughout isolation and characterization. It is noteworthy that even tropoelastin is difficult to isolate intact by using commonly reported isolation schemes. Mecham & Foster (1977) have shown that highly purified preparations of tropoelastin may often be degraded, because of proteinase activity that persists throughout isolation. Tropoelastin that is isolated either by the procedure of Sandberg *et al.* (1975) or by that of Foster *et al.* (1975) can be degraded on storage. In our hands, signs of proteolysis may be observed in spite of the presence of ionic detergents, denaturants (such as urea), short-chain alcohols, or even boiling elastin preparations for short duration. Only selected serine proteinase inhibitors appear to retard elastin proteolysis effectively (Mecham & Foster, 1977; Rucker *et al.*, 1978). This point requires emphasis in view of other recent reports that argue against the possibility of elastin precursors greater than 70000 daltons in size (Ryhänen *et al.*, 1978).

The nature of the extension peptides has not been fully assessed. What function the extension peptides serve with respect to the assembly of soluble elastin subunits into fibrous elastin is also not clear. Foster *et al.* (1978) have suggested that the extension peptides arise from both the *N*- and *C*-terminal ends of tropoelastin and are involved in the registration of tropoelastin to microfibrillar proteins in the initial assembly of insoluble elastin. Others including ourselves (Smith & Carnes, 1973; Narayanan & Page, 1976; Bressan & Prockop, 1977; Rucker *et al.*, 1977) have suggested that tropoelastin is the most immediate precursor to elastin in its cross-linked and insoluble form. However, these experiments were carried out by using conditions that would have precluded the detection of a proelastin. Whether the cleavage of a proform to tropoelastin represents a signal for the cross-linking of elastin or represents a signal for the eventual proteolysis of excess elastin (or other process) is another question that requires resolution. For example, we find it noteworthy that the putative proform to tropoelastin was observed as the predominant form *in vivo* (cf. Figs. 3, 4 and 5),

shown). However, a doublet near pH8.5 always appeared in isoelectric-focusing gels. Gel (b) is the final product obtained after isoelectric focusing, gel filtration, and alcohol and NaCl fractionation (see the text). The major band of protein (i) is focused at pH7.0. The diffuse band above it (ii) corresponds to pH10–10.5. It was also observed that some of the protein that was applied did not penetrate the gel. Band (iii) shows staining artifacts.

but appeared rapidly cleaved in culture *in vitro* (cf. Fig. 1).

Comments should also be made regarding the choice of starting material for these studies. Embryonic aorta and matrix-free smooth-muscle cells from several sources have been used for studies on elastin synthesis (Rucker & Tinker, 1977). We chose the young chick because during the period of 2 to 4 weeks post-hatching there is a rapid increase in the net amount of elastin that is synthesized by the aorta. At hatching, the elastin content of the aorta is approx. 5% of the wet weight. There is a gradual increase and then a very rapid synthesis of elastin commencing at 2 to 3 weeks post-hatching. By 4 to 5 weeks of age, approx. 14% wet wt. of the aorta is elastin (Lee *et al.*, 1976). The percentage of elastin in aorta does not change appreciably after this period. Relative to the synthesis of other proteins, as measured with [³H]-valine as a marker, it is easy to detect new elastin synthesis during the 2-4-week period. Under the conditions used here, it is extremely difficult to detect collagen or other protein synthesis relative to that for elastin (Lee *et al.*, 1976; Rucker *et al.*, 1977).

Although we have yet to characterize the putative proform to tropoelastin, some steps in its potential purification are suggested as a possibility for its eventual isolation and characterization. Isoelectric focusing appears to be a useful tool in separating the protein from other elastin-like peptides, and perhaps proteinases that are involved in its degradation. We are also certain that the proform to tropoelastin is not a post-extraction artifact. Its presence in arterial extracts has been described previously in a short communication (Rucker *et al.*, 1977) by using very rapid radiochemical labelling techniques and different extraction procedures. Furthermore in these experiments the extracts were subjected to reducing conditions and then carboxymethylated to preclude various interactions involving thiol-rich proteins (Rucker *et al.*, 1977). Modification of the currently used protocols for isolating soluble elastin (cf. Sandberg, 1976), particularly inclusion of appropriate inhibitors, should circumvent some of the problems associated with proteolysis, and result in elastin products that will provide new insights related to the initial stages of elastin fibrogenesis.

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