Lysyl Oxidase Activity and Elastin/Glycosaminoglycan Interactions in Growing Chick and Rat Aortas

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Abstract. Hydrophobic tropoelastin molecules aggregate in vitro in physiological conditions and form fibers very similar to natural ones (Bressan, G. M., I. Pasquali Ronchetti, C. Fornieri, F. Mattioli, I. Castellani, and D. Volpin, 1986, *J. Ultrastruct. Molec. Struct. Res.*, 94:209–216). Similar hydrophobic interactions might be operative in in vivo fibrogenesis. Data are presented suggesting that matrix glycosaminoglycans (GAGs) prevent spontaneous tropoelastin aggregation in vivo, at least up to the deamination of lysine residues on tropoelastin by matrix lysyl oxidase.

Lysyl oxidase inhibitors beta-aminopropionitrile, aminoacetonitrile, semicarbazide, and isonicotinic acid hydrazide were given to newborn chicks, to chick embryos, and to newborn rats, and the ultrastructural alterations of the aortic elastic fibers were analyzed and compared with the extent of the enzyme inhibition. When inhibition was >65% all chemicals induced alterations of elastic fibers in the form of lateral aggregates of elastin, which were always permeated by cytochemically and immunologically recognizable GAGs. The number and size of the abnormal elastin/GAGs aggregates were proportional to the extent of lysyl oxidase inhibition. The phenomenon was independent of the animal species. All data suggest that, upon inhibition of lysyl oxidase, matrix GAGs remain among elastin molecules during fibrogenesis by binding to positively charged amino groups on elastin. Newly synthesized and secreted tropoelastin has the highest number of free epsilon amino groups, and, therefore, the highest capability of binding to GAGs. These polyanions, by virtue of their great hydration and dispersing power, could prevent random spontaneous aggregation of hydrophobic tropoelastin in the extracellular space.

TROPOELASTIN is a highly hydrophobic polypeptide chain that is secreted mainly by fibroblasts and smooth muscle cells (8, 29). In the extracellular space tropoelastin molecules are deaminated on free epsilon amino groups of lysine residues by the enzyme lysyl oxidase and cross-linked into the unsoluble polymer elastin through a series of nonenzymatic steps that lead to the formation of pyridinium rings linking two adjacent molecules (9, 25, 33).

Isolated and purified tropoelastin molecules have been shown to exhibit a great tendency to aggregate in physiological solution and at temperatures in the physiological range, giving rise to supramolecular structures very similar to the natural polymer elastin (3, 4). The temperature-dependent spontaneous aggregation of tropoelastin in vitro has been attributed to its high hydrophobicity, and has been thought to play a role in elastin fibrogenesis in vivo (4, 7, 16, 22, 29). Actually, the mechanisms involved in elastin fibrogenesis in vivo are very likely more complicated. Proteoglycans and glycoproteins of the extracellular matrix seem to be implicated in some phases of the process (15, 26, 35). Growing elastin fibers are almost always surrounded by glycoprotein microfibrils, which have been thought to be involved in the orientation of the fibers (6, 28). Recently, it was suggested that matrix proteoglycans might play a role in elastin fibrogenesis (22). This hypothesis was mainly based on experiments showing that, upon inhibition of lysyl oxidase by beta-aminopropionitrile fumarate (BAPN)[1] in vivo, aortic elastic fibers grew by lateral apposition of roundish aggregates, which were always permeated by cytochemically recognizable glycosaminoglycans (1, 21). It was suggested that trapping of glycosaminoglycans among elastin was induced by the persistence on elastin of positive lysine amino groups offering binding sites for these negatively charged matrix macromolecules, and that elastin/glycosaminoglycan association could be a normal event during elastin fibrogenesis, which might maintain tropoelastin in solution in the extracellular space, at least up to the deamination of lysine epsilon amino groups by lysyl oxidase (21, 22).

The aim of the present work was to try to substantiate this hypothesis by analyzing (*a*) whether elastin/glycosaminoglycan interactions were specific to BAPN or could be the result of the inhibitory effect of the chemical on lysyl oxidase; (*b*) whether elastin/glycosaminoglycan association could be in some way related to the amount of free epsilon amino groups

1. *Abbreviations used in this paper*: AAN, aminoacetonitrile hydrochloride; BAPN, beta-aminopropionitrile fumarate; INAH, isonicotinic acid hydrazide; SCH, semicarbazide hydrochloride.

on elastin; and (*c*) whether the phenomenon was specific for chicks or could have a more general occurrence and significance.

For this purpose, a series of chemically different inhibitors of matrix lysyl oxidase were given to chicks and rats and the inhibitory efficiencies of the chemicals were compared with the ultrastructural modifications induced on the aortic elastin fibers.

The results clearly indicated that (*a*) all inhibitors of lysyl oxidase induced the formation of identical abnormal elastin aggregates permeated by glycosaminoglycans; (*b*) the phenomenon was directly related to the extent of inhibition of lysyl oxidase; and (*c*) the event was independent from the animal species.

All these data indicate that glycosaminoglycans have rather strong affinity for epsilon amino groups of lysine residues on tropoelastin and support the hypothesis that highly hydrated matrix polyanions might play a role in elastin fibrogenesis and prevent random tropoelastin aggregation in the extracellular space.

Materials and Methods

Chemicals and Treatments

Beta-aminopropionitrile fumarate (BAPN), aminoacetonitrile hydrochloride (AAN), semicarbazide hydrochloride (SCH), and isonicotinic acid hydrazide (INAH) (all from Sigma Chemical Co., St. Louis, MO) were used as inhibitors of matrix lysyl oxidase.

Groups of 20 chicks each were given diets containing 0.01, 0.025, 0.04, 0.05, 0.06, 0.08, and 0.1% wt/wt BAPN, or 0.1% wt/wt AAN, or 0.1 and 0.2% wt/wt SCH, or 0.1 and 0.2% wt/wt INAH for 7 d after hatching. On the eighth day all animals were weighed and killed by decapitation and aortas were quickly removed, measured, and processed for electron microscopical and biochemical studies.

INAH was also injected into the yolk sac of chick embryos. Treatments were performed by injection of 0.1 ml sterile Tyrode's solution containing 7.4 mg INAH per egg on day 16 of incubation. In two experiments a second injection was given on day 18 of incubation. Controls were injected with the same amount of sterilized Tyrode's. 50 chick embryos were used for each experiment. In all cases embryos were killed and the aortas removed 2 d after the last injection.

On the fourth day after delivery, breast-feeding female rats were given drinking water containing 0.2, 0.4, 0.6, and 0.8% wt/vol BAPN, or 0.2, 0.3, and 0.4% wt/vol AAN, SCH, or INAH. The maximum concentration of the chemicals allowing newborn rats to survive for up to 15 d was 0.4% BAPN, and 0.3% AAN, SCH, and INAH. In two experiments treatment with BAPN at the lowest concentration was prolonged for up to 3 wk. After 15 d of treatment, offspring were weighed and killed by decapitation and aortas were quickly removed and processed for electron microscopy.

Electron Microscopy

A single cross section, 0.5 mm thick, from each thoracic aorta was cut at the same distance from cephalobrachial branches in chickens and from heart in rats, carefully measured by an optical magnifier, and fixed. Fixation was performed in 2.5% glutaraldehyde (Fluka AG, Buchs, Switzerland) in Tyrode's physiological solution, pH 7.2, containing 0.1% toluidine blue O (Merck, Darmstadt, Federal Republic of Germany) for the visualization of matrix glycosaminoglycans (1, 31). Fixation was prolonged for 24 h at room temperature, and the specimens were washed in the same buffer containing 0.05% toluidine blue O for ~30 min and postfixed in 1% osmium tetroxide (Merck) in Tyrode's, pH 7.2, containing 0.05% toluidine blue O for 2 h at room temperature. Samples were dehydrated in ethanol and propylene oxide and embedded in durcupan (Fluka AG).

For immunocytochemical studies, a second cross section from the thoracic aorta of each animal was fixed in 1% glutaraldehyde and 0.2% picric acid for 24 h at room temperature (17). The specimens were dehydrated in alcohol and propylene oxide and embedded in durcupan. In all cases sections were carefully oriented cross-radially to the vessel. Thin sections were stained with uranyl acetate and lead citrate and observed in a Siemens Elmiskop IA and a Philips 400 T electron microscope, or treated for immunocytochemistry before being stained and observed.

Immunocytochemistry

Rabbit polyclonal antibodies raised against chondroitinase ABC-digested bovine nasal cartilage proteoglycans were used to localize chondroitin sulfate proteoglycans in the aortas. The antibodies were kindly furnished by Dr. A. Bertolotto (University of Turin, Turin, Italy) and were characterized for their affinity to the unsaturated disaccharide unit attached to the core protein after chondroitinase ABC digestion (2, 5). The indirect immunogold procedure was used (34). The proteoglycan-specific antibodies were revealed by gold-labeled goat anti-rabbit IgG serum (15 nm gold particles; Janssen Pharmaceutica, Beerse, Belgium). The procedure used was as follows. Ultrathin sections of both glutaraldehyde-fixed postosmicated and glutaraldehyde-picric acid-fixed unosmicated samples were collected on uncoated 200-mesh nickel grids, etched in 10% hydrogen peroxide for 10 min at room temperature, and digested with chondroitinase ABC (E.C.4.2.2.4., from *Proteus vulgaris*; Sigma Chemical Co.), 0.1 U/ml in 0.05 M Tris-HCl, pH 8.0, for 16 h at 4°C. After washing in distilled water and in 0.05 M Tris-buffered saline (TBS), pH 7.4, the grids were sequentially incubated in (*a*) 1/20 dilution of normal goat serum (Miles Laboratories, Naperville, IL) for 30 min at room temperature; (*b*) 1/600 dilution of specific rabbit antiserum for 18 h at 4°C; (*c*) 1/25 dilution of gold-labeled goat anti-rabbit IgG serum for 1 h at room temperature. Normal goat serum and specific rabbit antiserum were diluted in TBS containing 0.2% BSA (Sigma Chemical Co.), pH 7.4. Gold-labeled goat anti-rabbit serum was diluted in TBS containing 1% BSA, pH 8.2, and the same buffer was used for washing after steps *b* and *c*. Finally, the grids were rinsed in TBS, pH 7.4, and in distilled water, stained with 3% uranyl acetate in 50% ethanol and with lead citrate, and observed in the microscope.

Lysyl Oxidase Assay

The tritium release method described by Pinnel and Martin (25) and modified by Kagan et al. (11) was used.

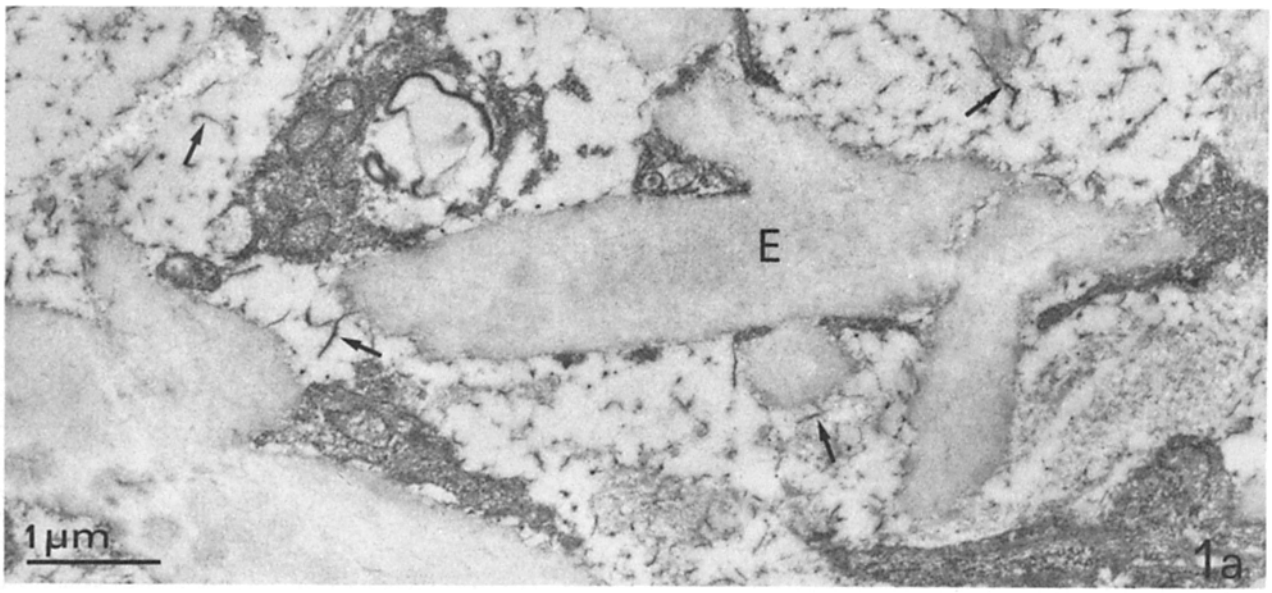
Briefly, aorta extracts containing lysyl oxidase were incubated with elastin-radiolabeled insoluble substrate and the amount of tritiated water produced by the enzymatic conversion of lysine residues to aminoadipic semialdehyde was measured as an expression of the enzyme activity.

Preparation of the Insoluble Substrate for Lysyl Oxidase

Freshly dissected aortas from 17-d-old chick embryos were collected and, in order to remove the endogenous soluble lysine, were rinsed for 30 min at 37°C in Dulbecco's and Vogt's modified Eagle's minimal essential medium, lacking lysine, flushed with O_2/CO_2 (19:1), and supplemented with 50 μg/ml of ascorbic acid, benzyl penicillin, and streptomycin sulfate, respectively.

The aortas were preincubated in stoppered Erlenmeyer flasks for 60 min at 37°C with gentle shaking at a ratio of 60 aortas for 20 ml of medium supplemented with 80 μg/ml BAPN in order to specifically inhibit endogenous lysyl oxidase. Then, after addition of 25 μCi/ml of L-[4,5-^{3}H]lysine hydrochloride (Amersham Corp., Arlington Heights, IL.), the tissue was incubated in the same conditions for 24 h. After incubation the medium was

Figure 1. Tonaca media of thoracic aortas from 7-d-old chicks. (*a*) Control. Elastin (*E*) formed long and branched fibers immersed in the extracellular matrix. The great majority of multibranched electron opaque filaments in the matrix should represent glycosaminoglycans revealed by the cytochemical stain toluidine blue O (*arrows*) (1, 31). (*b* and *c*) 0.1% BAPN in the diet for 7 d after hatching. Elastic fibers (*E*) showed lateral roundish appendices of lathyritic elastin (*LE*) permeated by toluidine blue O-stained glycosaminoglycans; In *c* glycosaminoglycans were visualized by the immunogold technique using polyclonal antibodies against the unsaturated disaccharide products of chondroitinase ABC-digested cartilage proteoglycans (2, 5). (*d*) 0.1% AAN and (*e*) 0.1% SCH added to the diet for 7 d after hatching. Elastic fibers (*E*) exhibited alterations and glycosaminoglycans interactions (*LE*) identical to those already observed after BAPN treatment.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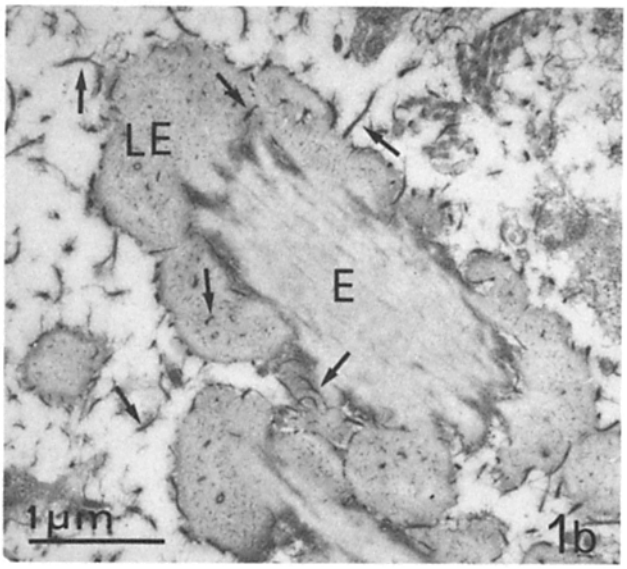
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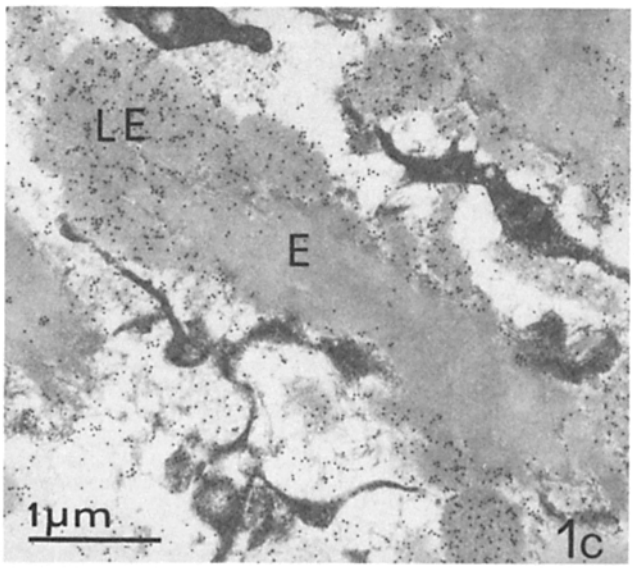
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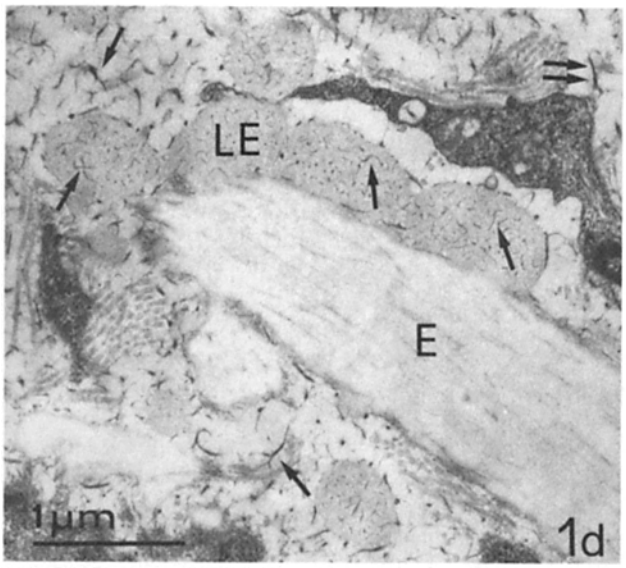
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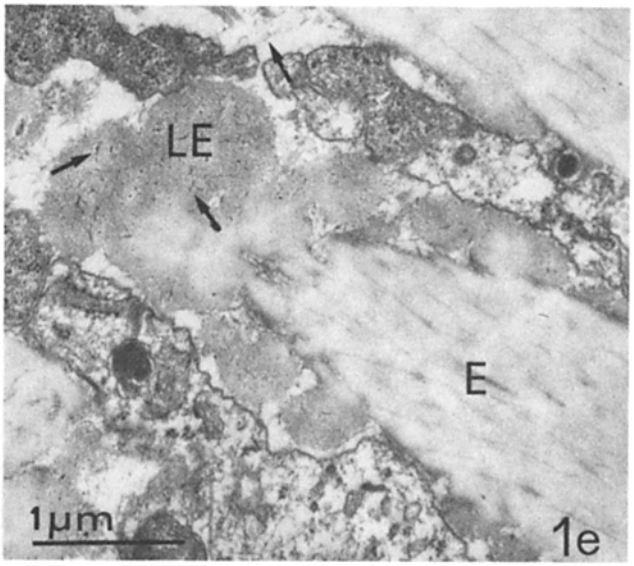
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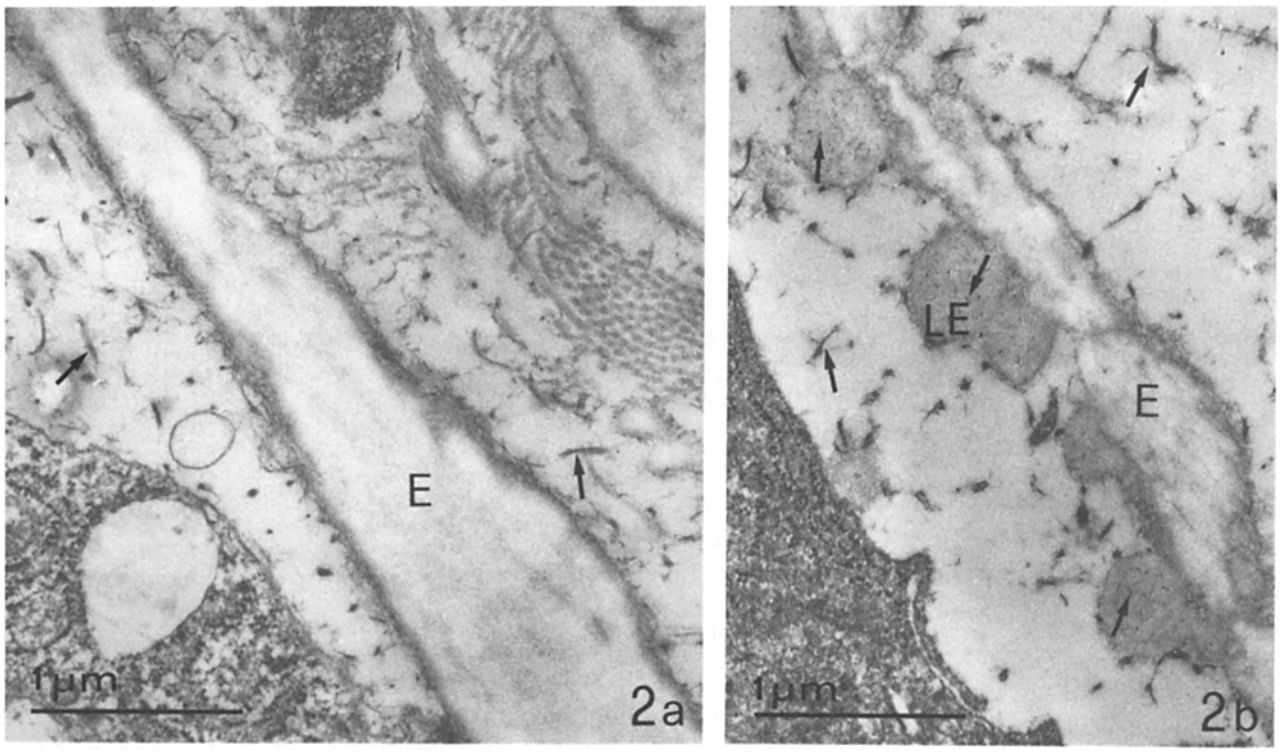

Figure 2. Tonaca media of thoracic aortas of (*a*) 7-d-old chick treated with 0.1% INAH added to the diet after hatching. (*b*) 18-d-old chick embryo after one injection of 7.4 mg INAH in 0.1 ml Tyrode's on day 16. INAH did not modify elastic fibers (*E*) when given to newborn chickens (*a*), whereas it induced alterations identical to those caused by all lysyl oxidase inhibitors when given to chick embryos (*b*). Glycosaminoglycans precipitates (*arrows*) were visible inside lathyritic elastin (*LE*) and in the matrix.

poured off and the aortas were washed with bidistilled water, suspended in a suitable volume of 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.4 (PBS), and homogenized in a Polytron (type PT10; Kinematica GmBH, Luzern, Switzerland) apparatus in an ice bath. The homogenate was centrifuged at 17,000 *g* for 30 min at 4°C, and the pellet suspended in 0.1 M PBS, homogenized again, and centrifuged as above. The final pellet, containing mainly elastin and collagen, was suspended in 0.1 M PBS (five aortas per milliliter), boiled for 10 min, and supplemented with 200 μg/ml BAPN to inhibit endogenous lysyl oxidase, stored at −80°C in 3-ml vials, and used as insoluble substrate for lysyl oxidase assay. The radioactivity of the substrate was checked before storing and before being used. The substrate was never stored for more than 2 mo.

Preparation of Lysyl Oxidase Source

Aortas from normal and treated chicks were quickly removed, accurately cleaned from adherent tissues, pooled from at least 15 chicks, frozen in liquid nitrogen, and powdered in a liquid nitrogen–cooled steel mortar. The following procedures were performed at 4°C. The aorta powder was added to 8 vol wt/vol of 4 M urea in 0.1 M Tris-HCl, pH 7.4, gently stirred for 4 h, and then centrifuged at 17,000 *g* for 30 min. The supernatant was exhaustively dialyzed against 0.1 M PBS overnight to remove urea, and used as a source of insoluble (or tightly bound to substrate in vivo) lysyl oxidase.

Lysyl Oxidase Assay

Two-tenths of a milliliter of suspension of the insoluble substrate, containing ~200,000 cpm, previously dialyzed overnight against 0.1 M PBS at 4°C to remove BAPN, was mixed with 0.5 ml of the enzyme preparation extracted from aortas, and 0.3 ml of 0.1 M PBS in a stoppered test tube. The mixture was then incubated for 5 h at 37°C in a shaking bath. Each assay was done in triplicate. It was assumed that any ^3H release during the incubation of the enzyme-substrate mixture represented the enzyme-dependent conversion of [^3H]lysyl to allysyl residues. The reaction was terminated by adding 0.1 ml of 50% trichloroacetic acid. 30 μl of octanol was added to each tube as an antifoaming agent.

Tritiated water formed during the reaction was collected by vacuum distillation and the radioactivity in 0.7 ml of the distillate was counted in 10 ml of liquid scintillant (Dynagel, Baker Chem BV, Deventer, Holland) by using a counter (Tri-Carb 2425; Packard Instrument Co., Downer's Grove, IL). The enzyme-dependent release of tritiated water was determined as the difference between tritiated water present in the incubation mixture and that present in the same mixture added to 100 μg/ml BAPN, which is an irreversible and specific inhibitor of lysyl oxidase (14).

The aortic lysyl oxidase activity of treated animals was expressed as a percentage of the activity in controls. As there was significant variation between batches of elastin substrate prepared at different times, care was taken to perform each complete experiment using the same substrate preparation.

Results

As specified in Materials and Methods, the inhibitors were added to the diet and given to chicks for 7 d after hatching. Only concentrations of the chemicals that allowed chickens to survive up to that time were considered. Nevertheless, the animals' condition had deteriorated by the end of the experiments; their weight was reduced by 22% with 0.1% BAPN, 30% with 0.1% AAN, 45% with 0.2% SCH, and 24% with 0.1% INAH compared with age-matched controls (mean of three experiments). Chicks showed leg dislocations, bone deformities, and sometimes, at autopsy, muscle hemorrhages.

The mean areas of aortic cross sections, measured on fresh specimens soon after removal, were also reduced; however, the ratios between body weight and aortic cross-sectional area were the same as in controls, indicating an impairment of growth of aortic tissue directly related to that of body weight.

The ultrastructural appearance of the aortic elastin fibers upon treatment with the various chemicals is reported in

Fig. 1. As already described (20, 21), aortic elastin of 7-d-old chicks formed long and branched fibers surrounded by a few electron-dense microfibrils (Fig. 1 *a*). Toluidine blue O added to the fixatives revealed glycosaminoglycans in the extracellular space in the form of multibranched electron-dense filaments (Fig. 1 *a*, *arrows*). 0.1% BAPN induced the elastin fibers to grow by apposition of roundish aggregates of elastin lateral to the fibers; moreover, these aggregates were always permeated by cytochemically (Fig. 1 *b*) and immunologically (Fig. 1 *c*) recognizable glycosaminoglycans. Identical features were observed upon treatment with 0.1% AAN (Fig. 1 *d*) or with 0.1% SCH (Fig. 1 *e*). INAH did not modify to an appreciable extent the ultrastructural appearance of aortic elastin fibers when given to newborn chicks (Fig. 2 *a*), whereas it induced alterations identical to those described for the other inhibitors when injected into the yolk sac of 16-d-old chick embryos (Fig. 2 *b*).

Fig. 3 reports the extent of inhibition of aortic lysyl oxidase activity by the various chemicals at the highest concentrations that allowed chickens to survive for up to 7 d. The inhibition was ~100% with BAPN, AAN, and SCH. INAH induced an inhibition not >65% when administered to newborn chicks, and always >80% when injected into the yolk sac of 16-d-old chick embryos.

To compare the extent of inhibition of lysyl oxidase activity with the alterations of elastin fibers, different concentrations of BAPN were given to chickens for 7 d after hatching. As shown in Fig. 4, the inhibition of aortic lysyl oxidase was directly related to the concentration of the drug in the diet. The ultrastructure of the elastic fibers of the aortic wall appeared normal with up to 0.025% BAPN in the diet, corresponding to 62% inhibition of lysyl oxidase activity. Then, by increasing the concentration of the chemical, elastin fibers started to exhibit lateral appendices, whose number and size, as well as amount of associated glycosaminoglycans, increased parallel to the increase of enzyme inhibition (Fig. 4).

The alterations of aortic elastic fibers induced by inhibitors of extracellular matrix lysyl oxidase were not specific to the species. BAPN, AAN, SCH, and INAH given to newborn rats through their mother's milk induced the aortic elastin fibers of the offspring to grow by apposition of lateral appendices of elastin permeated by cytochemically recognizable glycosaminoglycans, identical to those observed in chicks (Fig. 5, *a* and *b*). Lysyl oxidase inhibition could not be measured on rat aortas because we could not get enough tissue from each breeding to make confident evaluations of the enzyme activity.

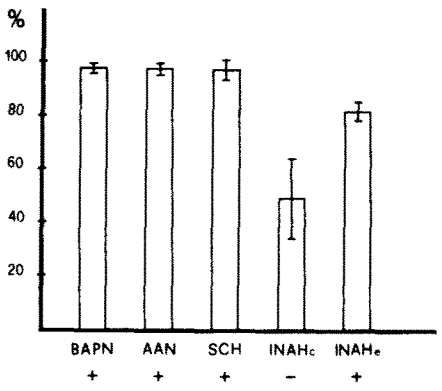

Figure 3. Percent inhibition of aortic lysyl oxidase activity induced by the various chemicals. The concentrations of the drugs, added to the diet for 7 d after hatching, were 0.1% BAPN and AAN, and 0.2% SCH. INAH was 0.2% when added to the diet (INAH c), and 7.4 mg/egg when injected into the yolk sac of chick embryos (INAH e) (see Materials and Methods). Mean of three experiments (±SD). (+) presence; (−) absence of glycosaminoglycans within lathyritic elastin.

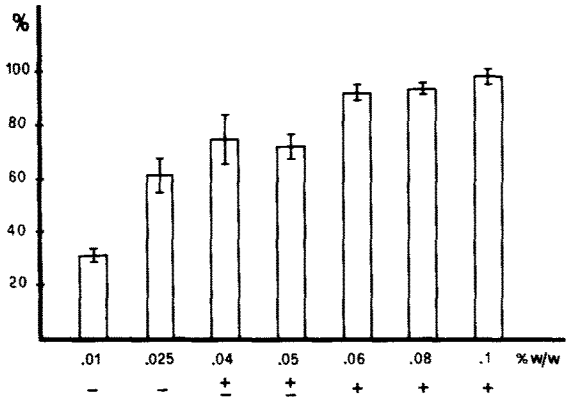

Figure 4. Effect of different concentrations of BAPN on lysyl oxidase activity of chick aorta. The chemical was administered for 7 d after hatching. Mean of three experiments (±SD). The percent inhibition of the enzyme activity was proportional to the amount of the chemical in the diet. The number and size of lateral appendices on elastin fibers as well as the amount of associated glycosaminoglycans were directly related to the extent of lysyl oxidase inhibition (plus signs at bottom).

Discussion

The alterations of elastin fibers already described in BAPN-induced lathyrism in chickens (20, 21) were found to be independent of the chemical nature of lysyl oxidase inhibitors and of the animal species. Moreover, they were found to be directly related to the concentration of the chemical in the diet and to its inhibitory effect on lysyl oxidase activity.

In previous reports, when describing the ultrastructural modifications of aortic elastin fibers in chicks upon administration of BAPN (22), we suggested that the strict association between lathyritic elastin and matrix glycosaminoglycans was due to the persistence of epsilon amino groups on elastin lysine residues offering positive charges for matrix polyanions. We also hypothesized that such electrostatic interactions could be normal events, active at least in certain tissues and in certain periods of intense elastin synthesis (12, 13), which would prevent newly synthesized tropoelastin molecules from spontaneous random aggregation far from growing elastic fibers. In fact, it is known that tropoelastin secreted by cells is highly hydrophobic and has a great tendency to aggregate in vitro by increasing temperature up to physiological values (3, 4, 7). In vivo, such a behavior would lead to the formation of numerous random elastin aggregates.

The results of the present study seem to confirm the above mentioned hypothesis. Elastin/glycosaminoglycan association doesn't depend on the chemical nature of the inhibitor of lysyl oxidase nor on its inhibitory mechanism (29),

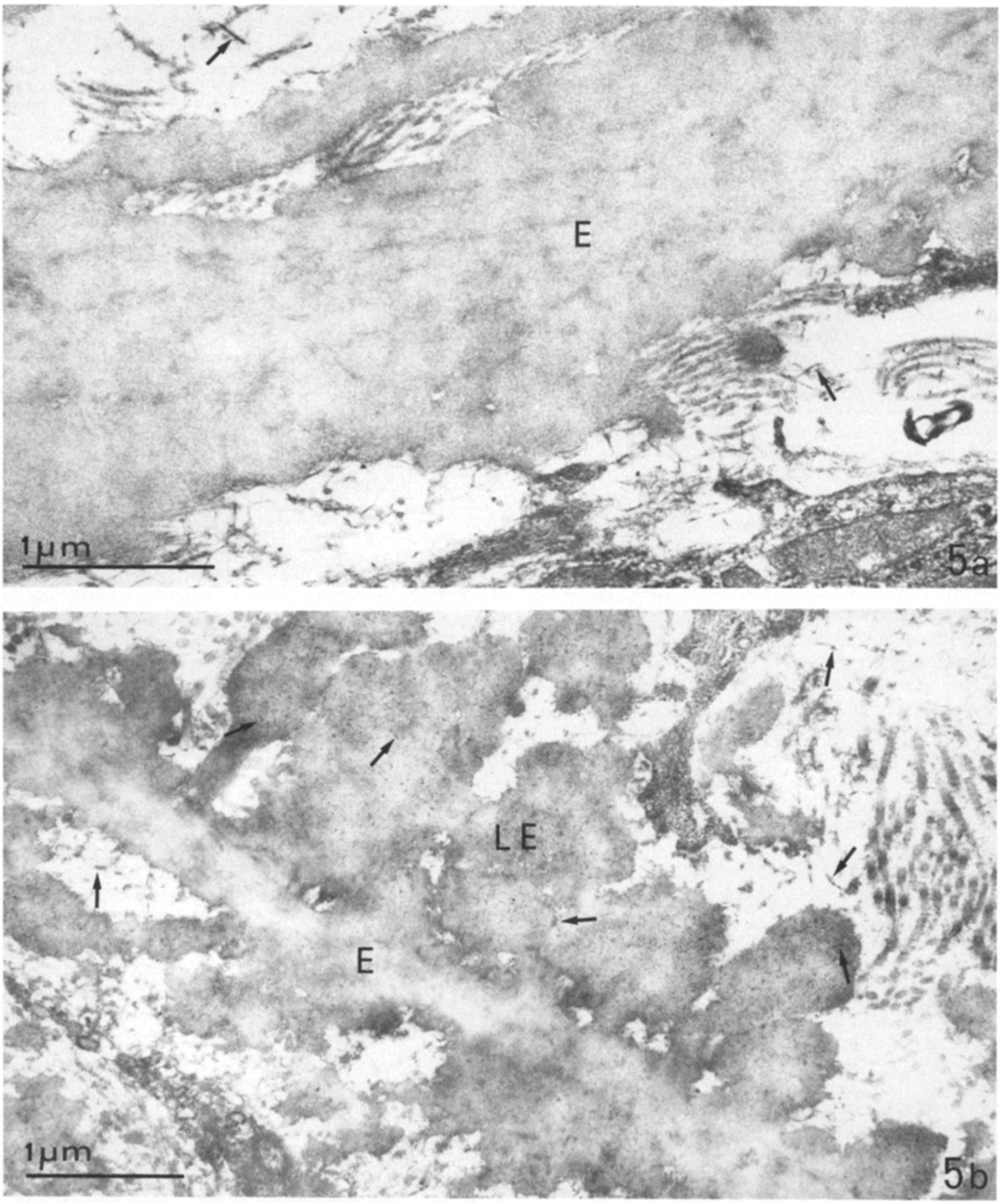

Figure 5. Tonaca media of thoracic aortas of 15-d-old rats. (*a*) Control. Elastin (*E*) formed linear lamellae circular to the lumen. In the extracellular space glycosaminoglycans were present in the form of long filaments stained with toluidine blue O (*arrows*). (*b*) 0.3% BAPN added to the drinking water of the rat mother (see Materials and Methods). The elastic fibers were deeply deformed by lateral appositions of lathyritic elastin (*LE*) permeated by toluidine blue O–stained glycosaminoglycans (*arrows*).

whereas it is quantitatively related to the extent of the enzyme inhibition.

It is interesting to note that INAH did not induce severe inhibition of lysyl oxidase; nor did it modify elastin fibrogenesis in newborn chickens. On the contrary, when given to chick embryos, it induced both severe lysyl oxidase inhibition and elastin/glycosaminoglycan association, as did all other inhibitors. The different behavior in embryos and chicks very likely depends on the difference in animal metabolism before and after birth (10). However, for the purpose

of the present study, the results suggest once more that elastin/glycosaminoglycan association is the result of the persistence of free epsilon amino groups on elastin.

It is also interesting to note that abnormal elastin fibers and elastin/glycosaminoglycan association were seen only when lysyl oxidase activity was markedly inhibited. In fact, no modifications of elastin fibrogenesis were observed upon treatment with low concentrations of lathyrogens, and in every instance the enzyme activity was <60% inhibited. Similar results were previously obtained upon treatment of the same animal model with penicillamine, which prevents desmosine formation by binding to precursors of the final cross-links (18, 19, 32), and was shown to inhibit by 30–50% the activity of aortic lysyl oxidase without inducing elastin/glycosaminoglycan interactions (23).

All these data strongly indicate that glycosaminoglycans are associated with elastin only when this molecule maintains a relatively high number of positive charges. The most obvious conclusion is that, since newly secreted tropoelastin molecules retain the highest number of positive charges, they have the highest capability of associating with matrix polyanions. Therefore, elastin/glycosaminoglycan interaction should be a normal event during active tropoelastin synthesis and secretion. Such an interaction would very likely help in dispersing tropoelastin molecules, thus preventing their spontaneous hydrophobic self-aggregation far from growing elastin fibers.

Whether these interactions are already operating inside the cell is under investigation.

Finally, the results of the present study seem to suggest that, independently of the animal species, alterations of aortic elastin fibers such as those described here could be considered good indices of severe lysyl oxidase inhibition or deficiency (24, 27, 30).

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References

1. Baccarani Contri, M., C. Fornieri, and I. Pasquali Ronchetti. 1985. Elastin-proteoglycan association revealed by cytochemical methods. *Connect. Tissue Res.* 13:237–249.
2. Bertolotto, A., L. Palmucci, A. Gagliano, T. Mongini, and G. Tarone. 1986. Immunohistochemical localization of chondroitin sulfate in normal and pathological human muscle. *J. Neurol. Sci.* 73:233–244.
3. Bressan, G. M., I. Castellani, G. M. Giro, D. Volpin, C. Fornieri, and I. Pasquali Ronchetti. 1983. Banded fibers in tropoelastin coacervates at physiological temperatures. *J. Ultrastruct. Res.* 82:335–340.
4. Bressan, G. M., I. Pasquali Ronchetti, C. Fornieri, F. Mattioli, I. Castellani, and D. Volpin. 1986. Relevance of aggregation properties of tropoelastin to the assembly and structure of elastic fibers. *J. Ultrastruct. Molec. Struct. Res.* 94:209–216.
5. Christner, J. E., B. Caterson, and J. R. Baker. 1980. Immunological determinants of proteoglycans. Antibodies against unsaturated oligosaccharide products of chondroitinase ABC-digested cartilage proteoglycans. *J. Biol. Chem.* 255:7102–7105.
6. Cleary, E. G., and M. A. Gibson. 1983. Elastin-associated microfibrils and microfibrillar proteins. *Int. Rev. Connect. Tissue Res.* 10:97–209.
7. Cox, B. A., B. C. Starcher, and D. W. Urry. 1974. Coacervation of tropoelastin results in fiber formation. *J. Biol. Chem.* 249:997–998.
8. Damiano, V., A. L. Tsang, G. Weinbaum, P. Christner, and J. Rosenbloom. 1984. Secretion of elastin in the embryonic chick aorta as visualized by immunoelectron microscopy. *Collagen Relat. Res.* 4:153–164.
9. Eyre, D. R., M. A. Paz, and P. M. Gallop. 1984. Cross-linking in collagen and elastin. *Annu. Rev. Biochem.* 53:717–748.
10. Freeman, B. M. 1978. Chemical embryology. *Chem. Zool.* Vol. X:76–106.
11. Kagan, H. M., N. A. Hewitt, L. L. Salcedo, and C. Franzblau. 1974. Catalytic activity of aortic lysyl oxidase in an insoluble enzyme-substrate complex. *Biochim. Biophys. Acta.* 365:223–234.
12. Keeley, F. W. 1979. The synthesis of soluble and insoluble elastin in chick aorta as a function of development and age. Effect of high cholesterol diet. *Can. J. Biochem.* 57:1273–1280.
13. Keeley, F. W., and D. J. Johnson. 1984. A comparison of the synthesis of elastin in aortic tissue of developing rats and chickens. *IX Meet. Fed. Eur. Connect. Tissue Soc. (FECTS), Budapest.* 80 (Abstr.)
14. Levene, C. I., and M. J. Carrington. 1985. The inhibition of protein-lysine-6-oxidase by various lathyrogens. Evidence for two different mechanisms. *Biochem. J.* 232:293–296.
15. Mecham, R. P., J. G. Madaras, and R. M. Senior. 1984. Extracellular matrix-specific induction of elastogenic differentiation and maintenance of phenotypic stability in bovine ligament fibroblasts. *J. Cell Biol.* 98: 1804–1812.
16. Narayanan, A. S., R. C. Page, F. Kuzan, and C. G. Cooper. 1978. Elastin cross-linking in vitro. Studies on factors influencing the formation of desmosines by lysyl oxidase action on tropoelastin. *Biochem. J.* 173:857–862.
17. Newman, G. R., B. Jasani, and E. D. Williams. 1983. A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. *Histochem. J.* 15:543–555.
18. Nimni, M. E. 1968. A defect in the intramolecular and intermolecular cross-linking of collagen caused by penicillamine. I. Metabolic and functional abnormalities in soft tissues. *J. Biol. Chem.* 243:1457–1466.
19. Nimni, M. E., K. Deshmuth, N. Gerth, and L. A. Bavetta. 1969. Changes in collagen metabolism associated with the administration of penicillamine and various amino and thiol compounds. *Biochem. Pharmacol.* 18: 707–714.
20. Pasquali Ronchetti, I., C. Fornieri, I. Castellani, G. M. Bressan and D. Volpin. 1981. Alterations of connective tissue components induced by beta-aminopropionitrile. *Exp. Mol. Pathol.* 35:42–56.
21. Pasquali Ronchetti, I., G. M. Bressan, C. Fornieri, M. Baccarani Contri, and D. Volpin. 1984. Elastin fibers-associated glycosaminoglycans in beta-aminopropionitrile-induced lathyrism. *Exp. Mol. Pathol.* 40:235–245.
22. Pasquali Ronchetti, I., M. Baccarani Contri, C. Fornieri, D. Quaglino Jr., and G. Mori. 1985. Alterations of elastin fibrogenesis by inhibition of the formation of desmosine cross-links. Comparison between the effect of beta-aminopropionitrile (β-APN) and penicillamine. *Connect. Tissue Res.* 14:159–167.
23. Pasquali Ronchetti, I., C. Fornieri, M. Baccarani Contri, D. Quaglino Jr., and E. Caselgrandi. 1986. Effect of penicillamine on the aorta of growing chickens. Ultrastructural and biochemical studies. *Am. J. Path.* 124:436–447.
24. Pasquali Ronchetti, I., D. Quaglino Jr., M. Baccarani Contri, R. Tenconi, G. M. Bressan, and D. Volpin. 1986. Aortic elastin abnormalities in Osteogenesis Imperfecta type II. *Collagen Relat. Res.* 6:409–421.
25. Pinnell, S. R., and G. R. Martin. 1968. The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptidyl linkages to allysine by an extract from bone. *Proc. Natl. Acad. Sci. USA.* 61:708–716.
26. Podrazky, V., S. Stokrova, and I. Fric. 1975. Elastin-proteoglycan interaction. Conformational changes of α-elastin induced by the interaction. *Connect. Tissue Res.* 4:51–54.
27. Rosenbloom, J. 1984. Elastin: relation of protein and gene structure to disease. *Lab. Invest.* 51:605–623.
28. Ross, R., and S. J. Klebanoff. 1971. The smooth muscle cells. I. In vivo synthesis of connective tissue proteins. *J. Cell Biol.* 50:159–171.
29. Rucker, R. B., W. Gottlich-Reimann, and K. Tom. 1973. Properties of chick tropoelastin. *Biochem. Biophys. Acta.* 317:193–201.
30. Sandberg, L. B., N. T. Soskel, and J. G. Leslie. 1981. Elastin structure, biosynthesis and relation to disease states. *New Engl. J. Med.* 304:566–579.
31. Shepard, N., and N. Mitchell. 1976. Simultaneous localization of proteoglycans by light and electron microscopy using toluidine blue O. A study of epiphyseal cartilage. *J. Histochem. Cytochem.* 24:621–629.
32. Siegel, R. C. 1977. Collagen cross-linking. Effect of D-penicillamine on cross-linking in vitro. *J. Biol. Chem.* 252:254–259.
33. Siegel, R. C. 1979. Lysyl oxidase. *Int. Rev. Connect. Tissue Res.* 8:73–118.
34. Varndell, I. M., F. J. Tapia, L. Probert, A. M. J. Buchan, J Gu, J. De Mey, S. R. Bloom, J. M. Polak. 1982. Immunogold staining procedure for the localization of regulatory peptides. *Peptides (Fayetteville).* 3: 259–272.
35. Velebny, V., M. Ledvina, and V. Lankasova. 1981. Interaction of a tropoelastin model with connective tissue components. *Connect. Tissue Res.* 9:63–68.