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Roles for Iron and Copper in Connective Tissue Biosynthesis

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Roles for iron and copper in connective tissue biosynthesis

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[Plate 1]

Both iron and copper play critical biochemical roles in the post-translational modifications of collagen and elastin. These modifications are essential to the maturation and structural integrity of these proteins. Iron functions in the hydroxylation of specific prolyl and lysyl residues in collagen, a process that must occur before the triple helix can form and be extruded from the cell. Copper functions in the oxidative deamination of specific lysyl residues in the soluble forms of both elastin and collagen. This process is essential for crosslink formation and the structural integrity of these proteins. While there is no evidence that nutritional iron deficiency results in connective tissue pathology, copper deficiency impairs crosslink formation and results in gross pathology of bones, lungs and the cardiovascular system of many animal species.

Introduction

Collagen and elastin constitute the major proteins of connective tissues. In vertebrates collagen makes up 25–30% of the total body protein, occurring in particularly high concentrations in bones, tendon and skin. While quantitatively less significant, elastin plays important structural and functional roles in ligaments and major arteries. The nutritionally essential elements, iron and copper, exert key catalytic functions in the post-translational biosynthesis and maturation of these proteins. This paper is concerned primarily with the biochemical and physiological roles of iron and copper, with a recognition of their nutritional interactions and relation to another nutrient, ascorbic acid.

Iron and ascorbic acid are involved in the hydroxylation of the connective tissue proteins, while copper is concerned with the maturation or crosslinking process. From a nutritional point of view, copper is essential for the absorption and mobilization of iron (Lee *et al.* 1968). Ascorbic acid enhances the absorption of non-haem iron (Sayers *et al.* 1973), but in excess it decreases the absorption of copper (Van Campen & Gross 1968). Nutritionally, these dietary components are interrelated in a triangular fashion as illustrated in figure 1.

The structure of collagen and of elastin

The collagen molecule, which provides high tensile strength and a degree of rigidity, is a coiled coil of three polypeptide units, known as α-chains. There are at least four types of collagen made up of different kinds and combinations of α-chains. Type I is the only type found in bone and tendon, and it accounts for about 90% of the collagen in man. The type I tropocollagen molecule contains two α1 chains, which are found only in type I collagen, and

one α2 chain, $[\alpha 1(I)]_2\alpha 2$. Glycine makes up about one-third of the amino acid residues in the α-chains and occurs as amino acid triplets, Gly-X-Y. Proline is the next most abundant amino acid and about one-third of the triplets have the sequence Gly-Pro-Y. The α1(I) chain contains about 1050 amino acid residues and has a relative molecular mass of approximately 100000. In the biosynthesis of collagen, cells first produce procollagen, a molecule whose α-chains are about 50% larger than those in tropocollagen. It is then processed to remove peptide extensions from both the amino and carboxy ends. The hydroxy amino acids, hydroxyproline and hydroxylysine, are almost unique to collagen and are essential to its structure (see reviews by Gallop & Paz (1975), Prockop *et al.* (1979) and Eyre (1980)).

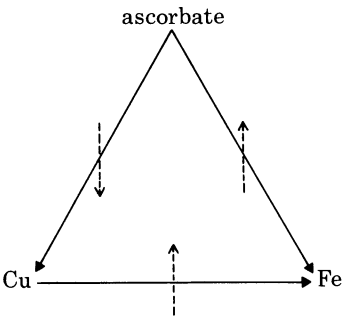

FIGURE 1. Nutritional interrelations of copper, iron and ascorbic acid. Copper is essential for iron absorption. Ascorbic acid enhances iron absorption but excess ascorbic acid depresses copper absorption. Broken arrows indicate positive or negative effects.

Mature elastin is a highly insoluble protein characterized by rubber-like elasticity. It is insoluble in almost all solvents that do not break covalent bonds. Insolubility results from extensive crosslinking and from its extremely hydrophobic nature. Elastin contains amino acids whose side chains are about 90% non-polar. As in collagen, glycine constitutes about one-third of the amino acid residues. The structure of mature elastin is not well defined, but it derives from the polymerization of a soluble subunit, tropoelastin, which has a relative molecular mass of about 70000. This protein accumulates in tissues in amounts that can be isolated only when crosslink formation is impaired, as it is in copper deficiency or lathyrism. Tropoelastin undergoes coacervation *in vitro* to produce an ordered structure consisting of filaments running parallel to the long axis of the fibre. The 3–5 nm filaments can be detected by electron microscopic examination of negatively stained specimens and has led several investigators to conclude that elastin is anisotropic. Recently, Aaron & Gosline (1980) have used polarized light microscopy to examine elastin fibres isolated from bovine ligaments. They observed extremely low residual birefringence and concluded that isolated elastin fibres are optically isotropic, in agreement with the kinetic theory of rubber elasticity. Details of elastin structure have been reviewed by Rucker & Tinker (1977) and Sandberg (1976).

So far as is known, iron and copper are concerned only with the post-translational modification of the polypeptide precursors. The first modification of both proteins involves hydroxylation.

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Hydroxylation

Type I collagen contains about 14% hydroxyproline or about 90 residues per 1000 residues. This is almost all 4-hydroxyproline. There is one residue of 3-hydroxyproline in α1(I), but other collagen types may contain 10 times as much of the 3-hydroxy isomer. Type I collagen contains an average of 6 residues of hydroxylysine per 1000 residues, whereas type IV collagen may have 8–10 times this concentration. Some of the hydroxylysine residues are glycosylated with galactose or a disaccharide of glucose and galactose. Elastin contains a smaller proportion of hydroxyproline than collagen, 10–15 residues per 1000, and no hydroxylysine.

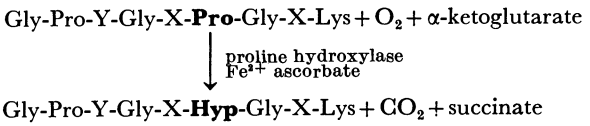

Figure 2. Hydroxylation of a collagen peptide catalysed by proline hydroxylase. Under the same conditions lysine hydroxylase would catalyse hydroxylation of the lysyl residue instead of the prolyl residue in the third position of the triplets.

Neither hydroxyproline nor hydroxylysine is incorporated directly into peptides and must arise by post-translational hydroxylation. Three different enzymes, prolyl-4-hydroxylase, lysyl hydroxylase and prolyl-3-hydroxylase, catalyse the hydroxylation of specific amino acid residues in α-chains before helix formation occurs. The first two enzymes act only on proline and lysine in the Y position of the Gly-X-Y triplet. All enzymes require Fe^{2+} and ascorbic acid as cofactors, and stoichiometric quantities of O_2 and α-ketoglutarate are involved in the reaction. The reaction catalysed by prolyl hydroxylase may be depicted as shown in figure 2 (Jackson 1978). The requirement for ascorbate is not absolute in that it can be replaced by other reducing agents. Nevertheless, it is the most efficient of several compounds tested and the most likely cofactor *in vivo*. Prolyl hydroxylase can catalyse a number of reaction cycles without ascorbate, but then hydroxylation ceases even though only 1–2% of the total Fe^{2+} has been oxidized. The reductant is probably involved in reducing enzyme-bound iron (Myllyla *et al.* 1978).

From the standpoint of the present discussion, ferrous iron is the key component of the hydroxylation process. When chick tendon cells are incubated in the presence of the iron chelator α,α′-dipyridyl, proline hydroxylation is severely inhibited (Jimenez *et al.* 1973). Under these conditions the non-hydroxylated protein, protocollagen, is not secreted normally and accumulates in the cell, presumably within the rough endoplasmic reticulum of fibroblasts. From these and similar experiments it has been concluded that proline hydroxylation is essential for triple helix formation at 37 °C. The triple helix form is required for normal cellular secretion of collagen (Prockop *et al.* 1979). Ascorbate is also essential for optimal collagen secretion by fibroblasts (Peterkovsky 1972). The collagen synthesized and excreted in the absence of ascorbate is not fully hydroxylated.

While there is good evidence that nutritional ascorbic acid deficiency in animals affects collagen metabolism (Barnes & Kodicek 1972), there is no evidence that nutritional iron deficiency has such an effect. This may relate to the fact that other pathological signs, such as severe anaemia, intervene before cellular iron levels become limiting for collagen hydroxylation. Bornstein (1967) fed weanling rats on an iron-deficient diet for 6 weeks, at which time the haematocrit had dropped to 31% from a control value of 48%. He then isolated a cyanogen

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bromide-cleaved peptide, α1-CB2, from skin collagen and found that the number of hydroxyproline residues were the same as in controls. Although this experiment suggests that iron deficiency in animals has no effect on collagen hydroxylation, it is possible that iron deprivation could affect other species and tissues, especially under more severe conditions of depletion.

Copper deficiency and connective tissue pathology

Copper deficiency in man and animals may arise from either nutritional deprivation or a genetic defect in copper metabolism. Menkes's disease is an X-linked genetic disease, manifested in male infants at or near birth. These patients accumulate copper in the intestinal mucosa but fail to mobilize it from these and other cells (Danks 1977). The Brindled allele of the mottled mouse mutant (Mo^{br}) provides an excellent animal model of the human disease (Hunt 1974). Both of these genetic diseases result from defective copper metabolism and exhibit connective tissue pathology very similar, if not identical, to that observed in nutritional copper deficiency. The most vulnerable organs include the cardiovascular system, the lungs and the bones. The major difference in pathology between genetic and nutritional copper deficiency relates to iron metabolism. While anaemia is often observed in nutritional copper deficiency in many species, it does not occur in the genetic copper-deficiency diseases.

Cardiovascular system

Perhaps the most dramatic sign of copper deficiency is the sudden rupture of the heart or a major artery. Angiorrhexis is usually preceded by extensive dissecting aneurysms. These signs were first observed in pigs (Shields *et al.* 1962) and chicks (O'Dell *et al.* 1961), but usually do not occur in even second generation sheep or in rats fed on low copper diets from weaning. However, rats reared by dams fed on copper-deficient diets and given the same diet for several weeks develop grossly enlarged hearts; some develop aneurysms of the heart or haemo-pericardium. Cardiac enlargement is also common in pigs and chicks. The literature related to cardiovascular pathology in nutritionally deficient simple-stomached animals has been reviewed (Carnes 1968; O'Dell 1976). Mills *et al.* (1976) have made similar observations in cattle fed on a low copper diet. The walls of major vessels were decreased in thickness and spontaneous rupture occurred, but the histological appearance of the elastic laminae differed from that observed in the pig. Spontaneous rupture of a major artery is frequently the terminal event in patients with Menkes's disease as well as in the mottled mouse (Danks 1977). Subdural haemorrhage occurs in patients with Menkes's disease as well as in copper-deficient pigs, chicks and newborn rats.

While the biochemical basis of the cardiac hypertrophy is not known, it is clear that pathology of the major arteries relates to a lower content of mature collagen and elastin than normal. The photomicrograph in figure 3, plate 1, illustrates histopathology in aortic tissue caused by copper deficiency. The biochemical defect relates to impairment of the maturation process, rather than to biosynthesis of the precursor polypeptides. Although the concentration of total collagen is unchanged in the aorta of the pig (Weissman *et al.* 1963) and the chick (O'Dell *et al.* 1966*a*), the proportion of soluble collagen is greatly increased. Insoluble elastin concentration is also decreased in the pig (Weissman *et al.* 1963) and chick aorta (Starcher *et al.* 1964; O'Dell *et al.* 1966*a*). The methods used for elastin determination remove the soluble elastin component and so there is no reliable estimate of its concentration in copper-deficient aortas.

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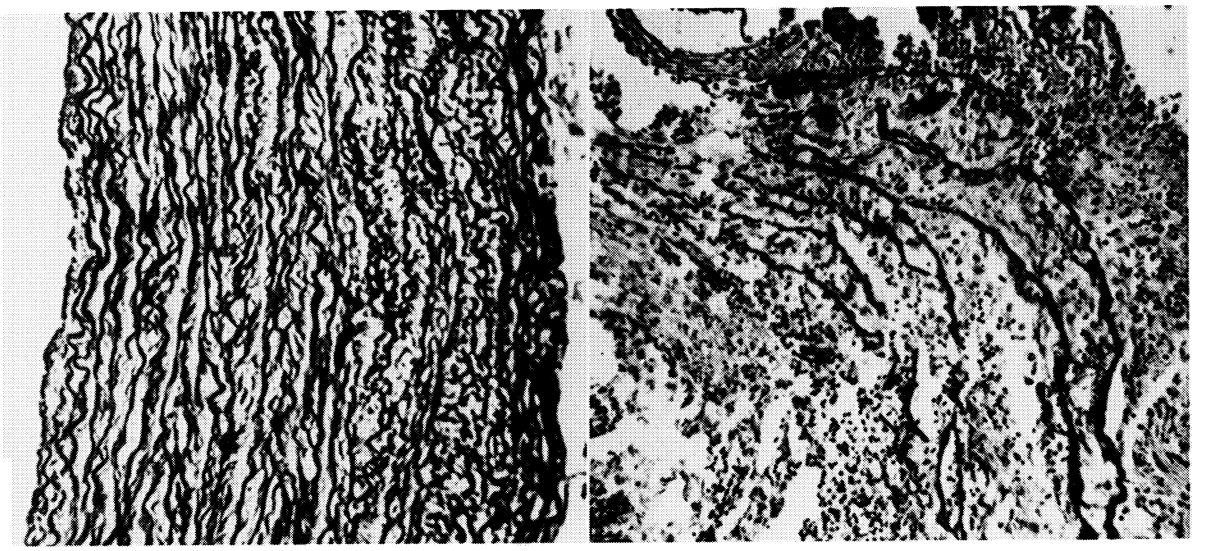

FIGURE 3. Histological sections of chick aortas: left, from a control; right, from a copper-deficient chick. Note fragmentation of elastic laminae and accumulation of material between laminae.

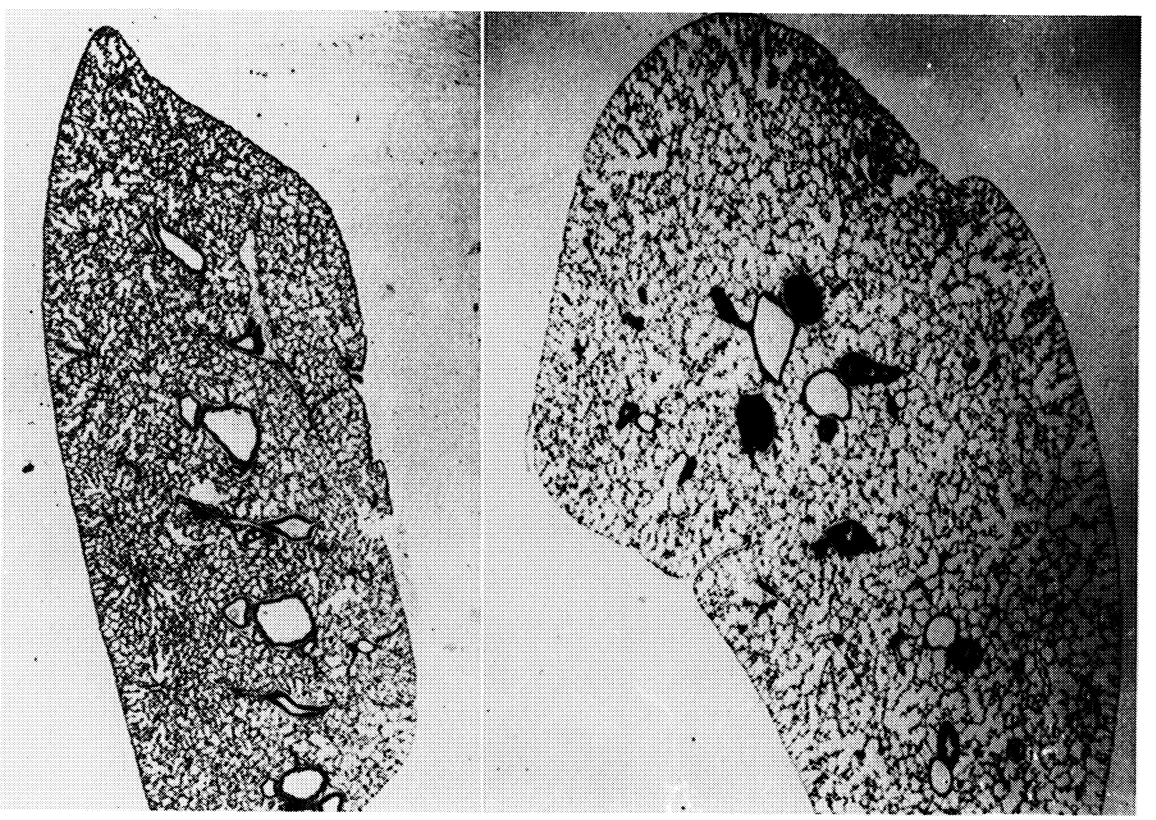

FIGURE 4. Sagittal sections of lungs from immature control (left) and copper-deficient (right) rats. Note the enlarged alveoli and alveolar ducts in the lung from the copper-deficient rat.

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Defective maturation of collagen offers a plausible, but unsubstantiated, explanation for the subcutaneous haemorrhage associated with copper deficiency.

Lung

The many cell types found in the lung parenchyma are supported by extracellular collagen and elastin, and the integrity of these proteins is essential for normal lung function. Pulmonary emphysema usually results from the destruction of the supporting structure, but also occurs owing to failure of its formation; developmental emphysema occurs in genetic as well as nutritional copper deficiency. Fisk & Kuhn (1976) have described emphysema-like changes in the lungs of the Blotchy mouse. This mottled mutant (Mo^{blo}) suffers from impaired copper absorption and metabolism, although to a lesser extent than in the Brindled mouse (Hunt 1974). The lung of the Blotchy mouse is structurally and functionally abnormal. The air spaces are enlarged and the lungs are more compliant than normal and have decreased elastic recoil. O'Dell *et al.* (1978) observed similar structural defects in second generation copper-deficient rats. The nature of the pathology is shown in figure 4, plate 1. The copper-deficient lungs had 35% larger alveolar spaces and contained significantly less elastin. Copper repletion during a period of 3 months did not restore the tissue to a normal condition.

Bone and tendon

Skeletal abnormalities due to copper deficiency have been observed in many species, including patients with Menkes's disease (Underwood 1977; Mills *et al.* 1976; Danks 1977). There is impairment of osteogenesis, with thinning of the cortex and trabeculae of long bones. Bones of copper-deficient animals are more fragile than normal, but the ash content is unaffected. Rucker *et al.* (1975) showed that bones from copper-deficient chicks fracture with less deformation and torque than do control bones. While there was no difference in the total collagen content of the dry, fat-free bones, the collagen in the deficient bones was more than three times as soluble in 5 M guanidine. These and earlier studies (see Rucker *et al.* (1975) for references) strongly suggest that impaired collagen maturation accounts for the bone defects associated with copper deficiency.

Specific pathology of tendons has not been observed in copper deficiency, but some gross pathology may result from defective tendons. The chemical nature of tendon collagen from copper-deficient chicks has been studied (Chou *et al.* 1968, 1969). To a large extent the degree of intermolecular crosslinking determines collagen solubility. As shown in figure 5, when chick achilles tendon is extracted successively with salt solutions and acetic acid, significantly more collagen is solubilized from copper-deficient than from control tendon. A similar effect was observed in aortic tissue, but not in skin. The degree of intramolecular crosslinking has been determined by separating the α and β chains of denatured soluble collagen on carboxymethylcellulose (Chou *et al.* 1969). The β_{11} component was not detectable in the copper deficient collagen, while the α1 component increased. The results suggest a failure of crosslink formation between α chains, as well as between molecules of collagen. Ganezer *et al.* (1976) showed that copper deficiency has no effect on tensile strength, elastic modulus or elastic recoil in pig tail tendon; the tensile strength of aortas from the same animals was impaired. These findings suggest that there is a species difference and tissue difference in crosslink formation, or that crosslinks are not the limiting factor in determination of tensile strength.

The gross and chemical pathology of copper deficiency strongly indicate defective cross-

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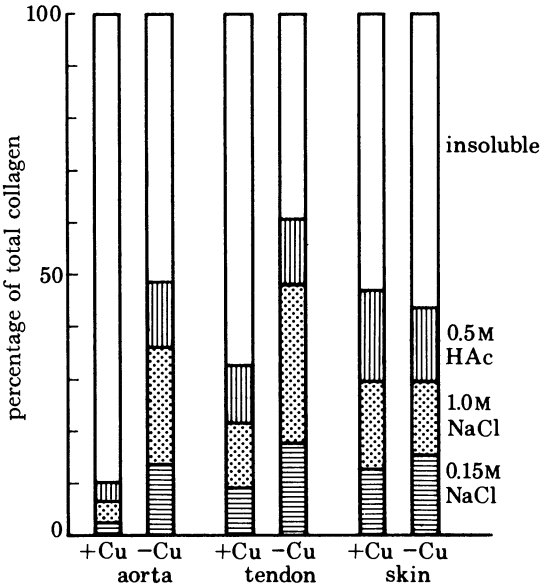

FIGURE 5. Effect of copper deficiency in chicks on the solubility of achilles tendon, aorta and skin collagen. Increased solubility in salt and acetic acid solutions indicates decreased intermolecular crosslinking in aorta and tendon collagen.

linking of both collagen and elastin. A brief description of the chemical nature of the crosslinks in these proteins follows.

ELASTIN CROSSLINKS

Although disulphide bonds have long been recognized to crosslink polypeptides, the first demonstration of the involvement of unique amino acids in crosslinks came from the laboratory of Partridge *et al.* (1963). Thomas *et al.* (1963) identified the two complex crosslinks in elastin as pyridinium derivatives and named them desmosine and isodesmosine. Other simpler compounds serve not only as crosslinking agents, but also as precursors in the biosynthesis of the desmosines. The crosslinking amino acids in elastin are listed in table 1. It is significant that

TABLE 1. MAJOR CROSSLINKS IN ELASTIN

(See Gallop *et al.* (1972) and Rucker & Tinker (1977). In parenthesis are the number of residues involved.)

amino acid	type of compound	peptidyl precursors
dehydrolysinonorleucine	aldimine	allysine (1); lysine (1)
lysinonorleucine	reduced aldimine; sec. amine	allysine (1); lysine (1)
aldol condensation product	α, β-unsat'd. aldehyde; dehyd. aldol	allysine (2)
dehydromerodesmosine	aldimine of lysine and aldehyde (above)	allysine (2); lysine (1)
merodesmosine	reduced aldimine (above)	allysine (2); lysine (1)
desmosine	pyridinium	allysine (3); lysine (1)
isodesmosine	pyridinium	allysine (3); lysine (1)

all are derived from lysine in peptide linkage. The key intermediate in their formation is the aldehyde derivative, α-aminoadipic acid δ-semialdehyde, also known as allysine. Francis *et al.* (1973) presented a possible scheme for the biosynthesis of the desmosines, involving the aldol condensation product and dehydromerodesmosine. Other mechanisms have been suggested (Rucker & Tinker 1977).

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Depending on species, uncrosslinked tropoelastin contains about 40 lysine residues, whereas mature elastin has about 5 residues per 1000 residues. The difference can be accounted for largely by lysine derivatives, which are absent from soluble elastin. There is evidence for 12 crosslinking sites per molecule of tropoelastin (Rucker & Tinker 1977); these regions are rich in alanine. Sequence data (Foster *et al.* 1974; Gerber & Anwar 1975) suggest that the desmosines link two peptide chains in regions such as are depicted in figure 6. Sequence data show that most tyrosine residues in soluble elastin follow lysine. It has been postulated that the ε-amino groups of these particular lysine residues supply the pyridine nitrogen of desmosine (Foster *et al.* 1974). Tyrosine may be the 'signal' that spares these particular lysines from the catalytic oxidation; subsequently they condense with allysine residues to form desmosine.

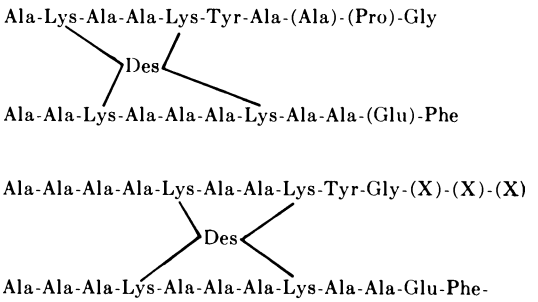

FIGURE 6. Amino acid sequences in elastin peptides containing desmosine (Foster *et al.* 1974).

Soluble uncrosslinked elastin accumulates in the aortas of copper-deficient animals to the extent that it can be isolated. Smith *et al.* (1968) first obtained soluble elastin from copper-deficient pigs, and it has since been isolated from aortas of copper-deficient chicks, calves and pigs. The isolation procedures have been reviewed by Sandberg (1976) and Rucker & Tinker (1977). Two peculiar properties of the protein have been useful in its purification. It is more soluble in aqueous alcohols than most proteins and, at 37 °C in the presence of high salt concentration, it undergoes coacervation.

The alkali-insoluble elastin isolated from aortas of copper-deficient chicks (Miller *et al.* 1965; O'Dell *et al.* 1966*b*) and pigs (Mecham & Foster 1979) contains lower concentrations of the desmosines than normal, whereas the lysine content is elevated. In pigs, dehydrolysinonorleucine and dehydromerodesmosine are higher in 'deficient' elastin than in normal elastin. The insoluble elastin isolated from copper-deficient pigs contained 10.6 residues of aldol condensation product per 1000, compared with 4–5 residues in normal bovine elastin (Lent *et al.* 1969). Copper deficiency impairs crosslink formation, and the primary site of the biochemical defect is the oxidative deamination of lysine to form allysine. It is not known whether there is selectivity in the oxidation of susceptible lysine residues in tropoelastin. Selectivity would account for the different distribution of the desmosine intermediates observed in the insoluble elastin isolated from copper-deficient animals. The primary oxidative reaction catalysed by the copper-dependent enzyme lysyl oxidase is discussed later. The activity of lysyl oxidase in tissues is depressed by nutritional copper deficiency and its activity in culture media of fibroblasts from patients with Menkes's disease is lower than normal (Royce *et al.* 1980).

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Collagen crosslinks

The recognition that lysyl residues serve as precursors of crosslinking amino acids in elastin quickly led to the identification of similar compounds in collagen. However, the desmosines do not exist in collagen. Table 2 lists the recognized crosslinks in collagen. Most of these compounds were identified after reduction with tritiated sodium borohydride. Lysinonorleucine and the aldol condensation product are common to both proteins, but hydroxylysine and its aldehyde derivatives, hydroxyallysine, play a more significant role in collagen crosslinking than lysine and its derivative. The chief reducible compound in bone and cartilage collagen is

Table 2. Major crosslinks in collagen

(See Gallop *et al.* (1972) and Tanzer (1973).)

amino acid	type of compound	peptidyl precursors
lysinonorleucine	reduced aldimine	allysine (1); lysine (1)
aldol condensation product (a.c.p.)	dehydrated aldol	allysine (2)
hydroxylysino-norleucine	reduced aldimine	HO-allysine (1)† and lysine (1)
dihydroxylysino-norleucine	reduced aldimine	HO-allysine (1) hydroxylysine (1)
hydroxymerodesmosine	reduced aldimine of a.c.p. and Hyl	allysine (2) hydroxylysine (1)
aldol-histidine (a.h.)	Michael addition product of a.c.p. and His	allysine (2) histidine (1)
histidino-hydroxy merodesmosine	reduced aldimine of a.h. and Hyl	allysine (2) histidine (1) hydroxylysine (1)

† Hydroxyallysine is δ-hydroxy-α-aminoadipic acid δ-semialdehyde.

dihydroxylysinonorleucine which is often glycosylated. This aldimine rearranges to the corresponding keto-amine, which is more stable. Histidine is also a constituent of the reduced collagen crosslinks (Tanzer 1973; Eyre 1980). Recently, a new crosslink has been postulated: Fujimoto *et al.* (1978) isolated a 3-hydroxypyridinium derivative from tendon collagen and named it pyridinoline. Eyre & Oguchi (1980) determined the concentration of this fluorescent compound in adult articular cartilage and found one residue per collagen molecule. This is more than 40 times the concentration of reducible crosslinks. However, Elsden *et al.* (1980) found that pyridinoline is not a crosslinking component of collagen; the compound could be removed from collagen by mild washing procedures and peptides of collagen did not contain it. Pending confirmation, pyridinoline cannot yet be considered as a collagen crosslink.

The reducible crosslinks found in neutral-salt and acid-soluble collagen are in the N-terminal and C-terminal non-helical regions of the molecule. Fukae & Mechanic (1980) have extended earlier observations that lysine residues in α1-chains at the 9^N (9th residue from N-terminus) and 17^C (9th residue from C-terminus) positions may be oxidized to allysine; the 6^N lysine in the α2-chain is also susceptible to oxidative deamination. These workers have now isolated two α1 chains, one of which contains a single aldehyde in the C-terminus ($\alpha 1^{lys,\ ald}$). The other chain contains two aldehydes ($\alpha 1^{ald_2}$) equally distributed between the N-terminal and C-terminal peptides. It was deduced that the carboxyl terminal aldehyde is synthesized first. During physiological maturation of skin and tendon collagen, the number of reducible crosslinks decreases to less than 10% of that in newly formed collagen. Apparently the reducible

crosslinks are modified to a non-reducible form. Cyanogen bromide digestion of mature bovine collagen yields a polymer of high molecular mass composed of the C-terminal (α1-CB6) and the N-terminal (α1-CB5) peptides (see Light & Bailey 1980).

The effect of copper deficiency on the concentration of specific collagen crosslinks has not been reported.

Lysyl oxidase

Pinnell & Martin (1968) first described an enzyme in embryonic chick bone that catalysed the oxidative deamination of peptidyl lysine in aortic tissue to form the allysyl residue. The assay was based on the rate of release of tritiated water from protein labelled by incorporation of [^{3}H]lysine. β-Amino-propionitrile (βAPN) at 0.04 mM inhibited the reaction maximally. This important observation has stimulated considerable research on the enzyme now known as lysyl oxidase; for a recent review see Siegel (1979).

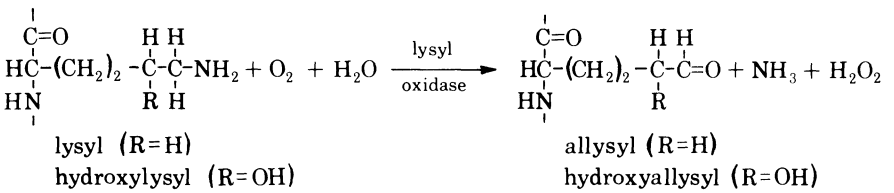

Figure 7. Reactions catalysed by lysyl oxidase. Peptidyl lysine and hydroxylysine are oxidatively deaminated to give the peptidyl allysine and hydroxyallysine.

Lysyl oxidase is a copper-dependent, extracellular amine oxidase catalysing the oxidation of specific lysyl and hydroxylysyl residues in elastin, collagen and probably other proteins such as those in eggshell membrane (Harris *et al.* 1980). The reactions catalysed are indicated by the equation in figure 7. This equation assumes that lysyl oxidase is a typical amine oxidase and that it catalyses the production of H_2O_2, ammonia and an aldehyde. There is no evidence that H_2O_2 is produced when peptidyl lysine serves as substrate, but Trackman & Kagan (1979) have shown that highly purified lysyl oxidase from bovine aorta produces H_2O_2 under some conditions. Thus it catalysed the oxidation of low molecular mass amines such as butylamine and non-peptidyl derivatives of lysine, and produced H_2O_2 and the corresponding aldehydes. The low molecular mass monoamines were competitive inhibitors of the reaction when insoluble aortic protein served as substrate. When tested as a substrate, βAPN did not give rise to a measurable quantity of H_2O_2, but it acted as a competitive inhibitor of the aortic protein as substrate. Sensitivity to βAPN inhibition has long been considered to be a property peculiar to lysyl oxidase. Narayanan *et al.* (1972) found that a large fraction of βAPN is irreversibly bound to lysyl oxidase when the enzyme in inactivated by βAPN in the absence of protein substrate. The data suggest that βAPN, simple monoamines and peptidyl lysyl residues compete for the active site on lysyl oxidase. However, this may be true only for selected types of the enzyme.

Lysyl oxidase is a copper-dependent enzyme and meets the criteria for a cuprometalloenzyme (Harris *et al.* 1974). Catalytic activity is decreased by metal chelators or acid precipitation, and it can be restored by cupric ions (Siegel *et al.* 1970; Harris *et al.* 1974). Nutritional copper deficiency in chicks decreases lysyl oxidase activity in cartilage (Siegel *et al.* 1970) and aortic tissue (Harris *et al.* 1974). Lung tissue from the Blotchy mouse possesses markedly less lysyl

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oxidase activity than normal (Starcher *et al.* 1977), as also do cultures of Menkes's fibroblasts (Royce *et al.* 1980). The activity in aortas of copper-deficient chicks is restored by administration of copper *in vivo*, but activation is blocked by cycloheximide (Harris 1976). Rayton & Harris (1979) showed that cupric ions will reactivate aortic tissue in culture under conditions that allow oxidative metabolism to proceed. Copper in the form of a serum protein complex was effective at lower concentration than copper in the ionic form. By the use of [^{3}H]lysine and ^{64}Cu it was shown that copper is bound to a newly synthesized protein of relative molecular mass about 60000. The results suggest that copper activation of lysyl oxidase involves the synthesis of the enzyme *de novo*, copper being incorporated during biosynthesis, rather than by addition to a preformed apoenzyme.

Lysyl oxidase has been isolated from collagenous tissue, chick bone cartilage and tissues rich in elastin, such as bovine ligamentum nuchae, bovine aorta and chick aorta (see Siegel 1979). The enzyme exists in multiple forms probably differing in charge, as three to four peaks of activity can be resolved by fractionation on DEAE-cellulose. Most isolation procedures use a

TABLE 3. RELATIVE MOLECULAR MASSES OF LYSYL OXIDASE

source	approx. M_r	references
chick cartilage	60000	Siegel & Fu (1976)
chick cartilage	28000	Stassen (1976)
chick aorta	60000	Harris *et al.* (1974)
bovine aorta	60000	Vidal *et al.* (1975)
bovine aorta	30000	Jordan *et al.* (1976)
bovine ligament	30000	Kagan *et al.* (1979)

combination of extraction with 4 M urea, affinity chromatography, DEAE-cellulose fractionation and gel permeation. As shown in table 3, the assigned relative molecular masses differ widely, but one group of values is about 30000 and the other about 60000; there is no obvious explanation for the difference. All investigators used sodium dodecyl sulphate polyacrylamide electrophoresis and Coomassie blue staining of protein bands to determine relative molecular mass. In addition, Jordan *et al.* (1976) and Kagan *et al.* (1979) used gel permeation of catalytic activity to measure molecular size and obtained good agreement between the two methods. The results suggest the existence of a dimeric form as well as a protomer with M_r = 30000, but they could be explained by association of a non-catalytic protein with the enzyme.

Stassen (1976) and Siegel & Fu (1976) found that all fractions of the cartilage enzyme eluted from DEAE-cellulose with a salt gradient have the same relative activity with a purified calvaria collagen substrate and the commonly used 'elastin' substrate. The latter is a crude preparation of insoluble proteins from embryonic chick aorta. The lack of substrate specificity of lysyl oxidase for proteins as different as collagen and elastin is an enigma. Recent work in our laboratory (Losty & O'Dell 1980) shows that extracts of bovine ligamentum nuchae can be separated by gel permeation into fractions that possess differential activity towards soluble elastin and soluble calvaria collagen as substrates. Urea extracts of the ligament were first fractionated on DEAE-cellulose (Harris & O'Dell 1974). In this procedure the column is first eluted with 0.5 M NaCl until most of the protein and associated enzyme activity are removed (fraction A); this is followed by a salt gradient in 6 M urea. The combined activity eluted with urea is here designated fraction B. It contained relatively little protein and a major proportion of the activity.

Fraction A was then eluted from a calibrated Sephadex G-100 column equilibrated with

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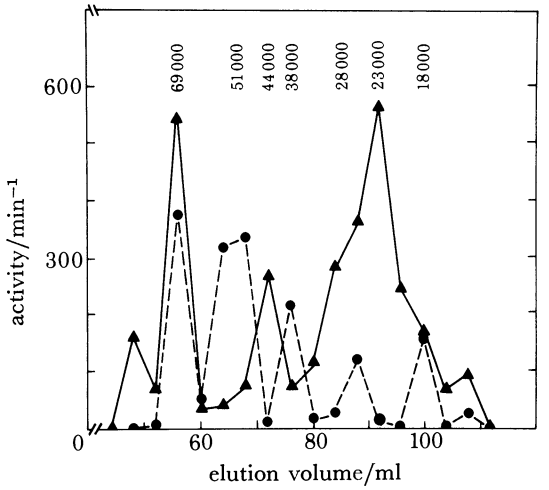

FIGURE 8. Lysyl oxidase activity profiles of ligamentum nuchae fraction A assayed with soluble collagen (– – –) and soluble elastin (——). Fraction A, which was eluted from DEAE-cellulose with 0.5 M NaCl, was filtered through Sephadex G-100 equilibrated with 4 M urea. Fractions of 2 ml were collected and alternate fractions assayed with calvaria collagen (450 000 disintegrations/min) and soluble aortic elastin (170 000 min^{-1}). The activity is that released by a 0.2 ml aliquot in a 1.0 ml assay volume.

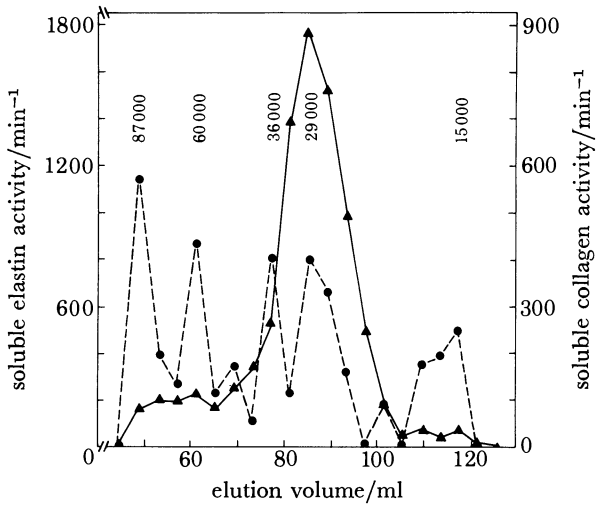

FIGURE 9. Lysyl oxidase activity profiles of ligamentum nuchae fraction B assayed with soluble collagen (– – –) and soluble elastin (——). Fraction B was eluted from DEAE-cellulose after fraction A with 0.5 M NaCl and 6 M urea. Other conditions as in figure 8.

4 M urea. Fractions were assayed with soluble collagen and soluble elastin as substrates. The activity profiles are shown in figure 8. With the soluble elastin substrate, peak activities occurred in tubes corresponding to relative molecular masses of 69 000, 44 000 and 23 000. The collagen substrate gave other peaks at M_r = 51 000, 38 000, 28 000 and 18 000. The comparable profile of fraction B on the same column is shown in figure 9. Activity with the soluble elastin substrate was eluted as one major peak at M_r = 29 000. With soluble collagen only one activity peak corresponded to the soluble elastin peak. Others occurred at M_r = 87 000, 60 000, 36 000 and 15 000. It is notable that the M_r = 69 000 form in fraction A acted on both collagen and elastin. The 60 000 and higher relative molecular mass components of fraction B catalysed the oxidation

of collagen only. These results suggest multiple molecular mass forms of the enzyme with different substrate specificities. This is contrary to what has been observed with more highly purified enzyme preparations. The results may be explained by polymeric forms of the enzyme in 4 M urea or by association of the enzyme with other proteins. In the absence of urea the enzyme polymerizes to form active units of extremely high molecular mass (Kagan *et al.* 1979). This no doubt occurs in the assay medium in the presence of a low concentration of urea. Nevertheless the different forms in 4 M urea show differential activity with the two substrates. It may be postulated that tissues contain modifiers of lysyl oxidase that change its substrate preference, if not its specificity.

Besides the substrate specificity question there is another unsolved problem related to lysyl oxidase, namely the assay for the unpurified enzyme as it occurs in tissues. From our experience and that of others, it is clear that crude extracts of many tissues contain an 'inhibitor' or 'inhibitors' which prevents linear response to increasing concentration of the extract. Little information relative to this problem has been published, but Rowe *et al.* (1977) have described it clearly. The addition of a skin extract to a purified enzyme preparation caused about 90% inhibition of the activity. This could be due to an inhibitor or to dilution of the radioactive substrate used in the assay by unlabelled substrate present in the crude enzyme extract. Purification of the extracts by affinity chromatography and/or DEAE-cellulose chromatography offers a partial solution, but recovery of activity during purification would then not be known.

Conclusion

At the cellular level, both iron and copper perform essential metabolic roles in the biosynthesis of mature collagen and elastin. In the whole animal only deficiency of copper has been shown to impair connective tissue metabolism and lead to gross pathology. Crosslinking of collagen and elastin may be the first limiting metabolic process for the survival of some animal species when they are deprived of copper. Although iron plays a key role in the post-translational modification of collagen, other metabolic processes appear to be more limiting in animals deprived of iron. Hence, iron-deficient animals succumb to anaemia before there are signs of impaired connective tissue metabolism.

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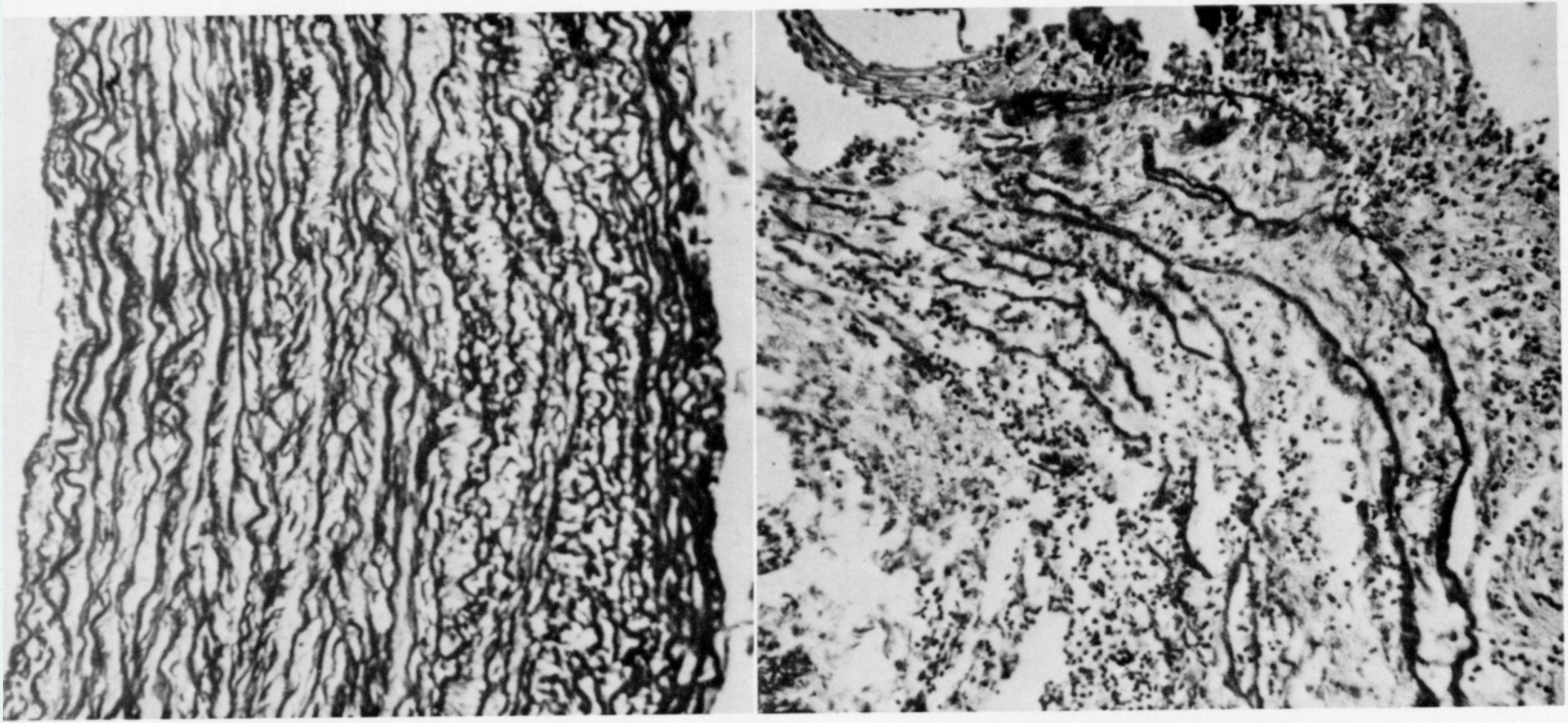

FIGURE 3. Histological sections of chick aortas: left, from a control; right, from a copper-deficient chick. Note fragmentation of elastic laminae and accumulation of material between laminae.

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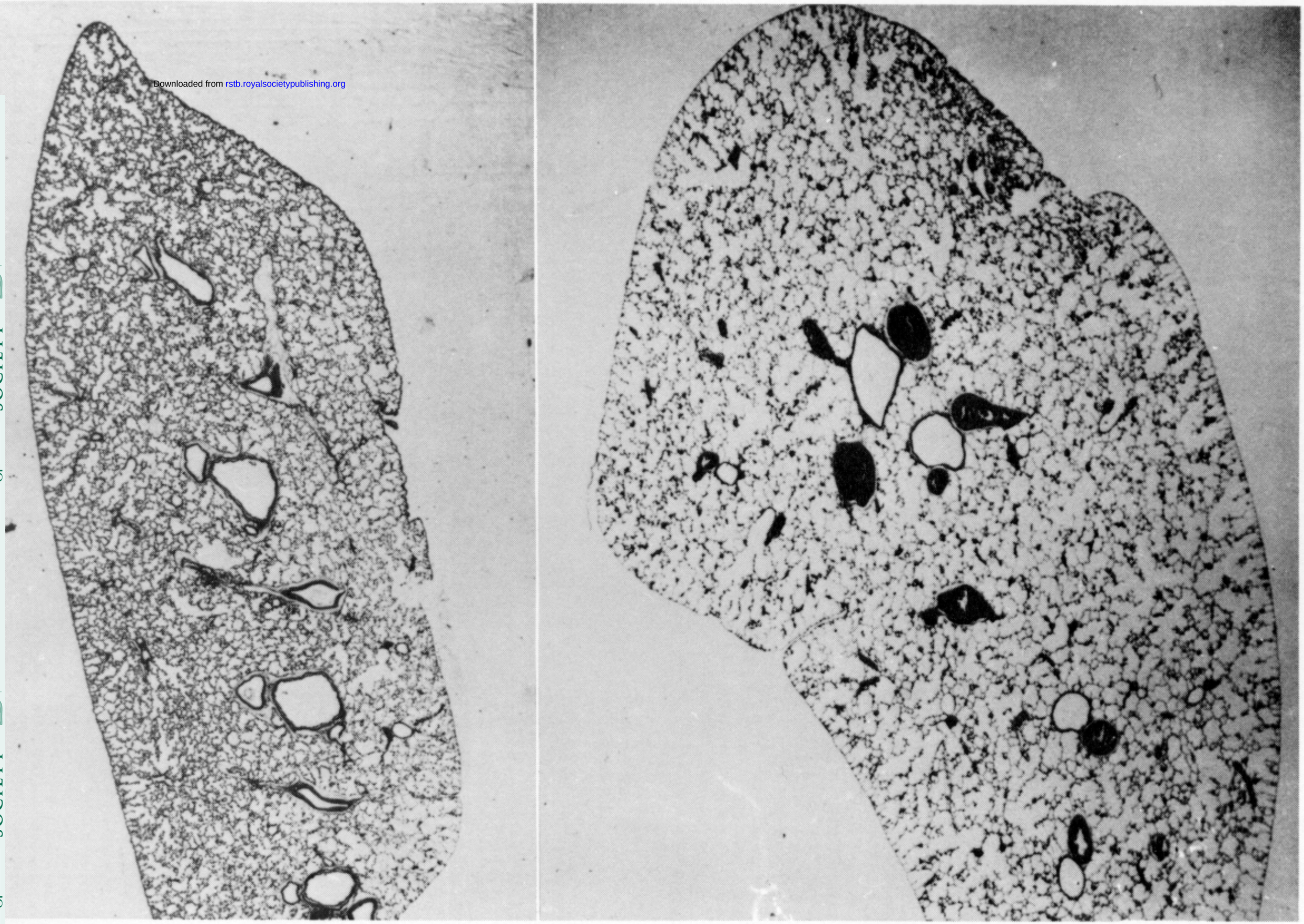

GURE 4. Sagittal sections of lungs from immature control (left) and copper-deficient (right) rats. Note the enlarged alveoli and alveolar ducts in the lung from the copper-deficient rat.