Biochemical and Molecular Roles of Nutrients

Modulation of Lysyl Oxidase by Dietary Copper in Rats

ROBERT B. RUCKER,* NADIA ROMERO-CHAPMAN,* TONIEL WONG,* JILL LEE,* FRANCENE M. STEINBERG,* CARL MCGEE,* MICHAEL S. CLEGG,* KAREN REIERVER,† TARU KOSONEN,* JANET Y. URIU-HARE,* JEROME MURPHY** AND CARL L. KEEN*

Departments of *Nutrition (College of Agriculture and Environmental Sciences), †Pulmonary Medicine and **Pediatrics (School of Medicine), University of California, Davis, CA 95616

ABSTRACT Lysyl oxidase levels were estimated in rat tissues using an enzyme-linked immunosorption assay (ELISA) and a functional assay standardized against known amounts of purified lysyl oxidase. High concentrations of lysyl oxidase (≥150 μg/g of tissue or packed cells) were detected in connective tissues, such as tendon and skin. Values for aorta, kidney, lung and liver ranged from 30 to 150 μg/g of tissue; values for skeletal muscle and diaphragm were <30 μg/g tissue. Purified rat skin lysyl oxidase catalyzed the release of 50–100 Bq of tritium per μg enzyme in assays that used 3H-elastin-rich substrates. In dense connective tissues, good agreement was obtained for the values from ELISA and those derived from measurements of functional activity in aorta, lung, skin and tendon (r² > 0.9). When egg white-based experimental diets containing 2 or 10 μg/g added copper were fed to weanling rats, values for skin lysyl oxidase functional activity in the group fed 2 μg/g added copper were one-third to one-half the values for skin lysyl oxidase functional activity in rats fed 10 μg/g copper. This reduction in lysyl oxidase activity, however, had minimal effect on indices of collagen maturation in rat skin, e.g., collagen solubility in neutral salt and dilute acid or the levels of acid stable cross-links. Moreover, copper deficiency did not influence the steady-state levels of lysyl oxidase specific mRNA in rat skin or the apparent amounts of lysyl oxidase in rat skin as determined by ELISA. These observations underscore that the concentration of lysyl oxidase is relatively high in dense connective tissues, and although decreasing dietary copper influences functional activity, there is little apparent effect on the production of lysyl oxidase protein. J. Nutr. 126: 51–60, 1996.

INDEXING KEY WORDS:
• copper • lysyl oxidase • collagen • elastin • rats

Lysyl oxidase [EC 1.4.3.13] is a copper-containing enzyme that catalyzes the oxidative deamination of lysyl or hydroxylysyl residues to corresponding peptidyl aldehydes (Kagan and Trackman 1991, Reiser et al. 1992). The process is novel because it occurs extracellularly and is the first step in a complex series of reactions that eventually result in the covalent cross-linking of collagen or elastin. Moreover, lysyl oxidase or a closely related protein may also serve as a ras antioncogene, i.e., a ras recession gene [rgr]. Kenyon et al. (1993) have shown that rgr from NIH 3T3 cells shares >85% identity with rat and human lysyl oxidase (also see Mariani et al. 1992).

Lysyl oxidase has been isolated and characterized from a number of sources: chicken cartilage (Stassen 1976), bovine lung and aorta (Cronlund and Kagan 1986), rat lung, placenta and skin (Almassian et al. 1990, Romero-Chapman et al. 1991), turkey aorta (Narayan et al. 1982), human placenta (Kuivaniemi 1985) and porcine skin (Shackleton and Hulmes 1990). Lysyl oxidase is often isolated as four isoforms, each with a molecular weight of 32 kDa. The enzyme also copurifies with other extracellular matrix proteins, such as tyrosine-rich acidic matrix protein (Cronshaw et al. 1993).

Trackman et al. (1990, 1992) and Romero-Chapman et al. (1991) reported that lysyl oxidase undergoes...

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3 To whom correspondence should be addressed.

4 Abbreviations used: DES, deamosine; DHLNL, dihydroxylysinoorniroleucine, ELISA, enzyme-linked immunosorption assay; HLNL, hydroxy-lysinoorniroleucine; Ide, isodesmosine; rgr, ras recession gene; RT-PCR, reverse transcription-polymerase chain reaction.
posttranslational processing that includes glycosylation and proteolysis. Prolysyloxidase is 50 kDa and is eventually processed to an active 32 kDa form of the enzyme. Although it has been established that human and mouse lysyl oxidase genes are located on chromosomes 5 and 18 as a single gene (Chang et al. 1993, Contente et al. 1993, Häimäinen et al. 1991), respectively, many of the principal factors involved in lysyl oxidase regulation have not been defined.

Reported here are studies in which lysyl oxidase activity in skin was examined using rats fed diets that are either deficient or sufficient in dietary copper. The levels of lysyl oxidase mRNA, lysyl oxidase protein and lysyl oxidase functional activity were examined in response to varying dietary copper. We also report the levels of lysyl oxidase in selected tissues as estimated by enzyme-linked immunosorption (ELISA) and in functional assays. Lysyl oxidase is present in relatively high concentrations in some tissues, and as a consequence, may constitute a substantial reservoir for tissue copper.

MATERIALS AND METHODS

Materials. Radioisotopes [L-3H-4,5-lysine, Sp. Act., 3.7 GBq/mmol and NaBH₄[3H], Sp. Act 300 GBq/mol] are products of Amersham Corporation (Arlington Heights, IL). Culture media were purchased from Grand Island Biochemical Company (Grand Island, NY). Reagents and supplies for antibody preparations were obtained from Antibodies (Woodland, CA), Ribi Immuno Chemical Research (Hamilton, MT), BioRad (Richmond, CA) and Pharmacia (Piscataway, NJ). The electrophoretic and column-chromatographic supplies are products of BioRad (Richmond, CA). Actigel-Ald was obtained from Sterogene (Arcadia, CA) and nitrocellulose from Schleicher and Schuell (New York, NY). All other chemicals were of the highest quality commercially available from Fisher Scientific (Pittsburgh, PA), Pierce Chemical (Rockford, IL), Aldrich Chemical (Milwaukee, WI) or Sigma Biological (St. Louis, MO).

Animal models and diets. Experiment 1. Virgin female Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing approximately 200 g were fed semipurified egg white protein-based diets containing 2 or 10 μg of copper as CuSO₄·5H₂O per g of diet. The diet composition and protocols are described in Chung et al. (1988). The animals were bred and the diets were fed throughout gestation and lactation. Resulting pups were weaned to the diet fed to the dam (2 or 10 μg of Cu per g diet). The goal was to partially deplete rats of copper without significantly influencing growth. Dorsal skin samples were used as the tissue source for the lysyl oxidase assays. Rats (4 per group) were killed by CO₂ inhalation at 1, 4 or 8 wk of age for the various assays. These experiments were reviewed by the UC Davis Animal Use Research Committee and comply with ALAACC, NIH, and USDA guidelines.

Experiment 2. Two groups of weaning Sprague-Dawley male rats (40–50 g initial body weight) were fed diets (see Chung et al. 1988) for the composition containing either <0.5 μg Cu/g (basal) or 10 μg/g (designated the control) for 6 wk. A primary goal was to assess whether a severe alteration in copper status influences the expression of skin lysyl oxidase mRNA transcripts in vivo.

Assay for lysyl oxidase. Lysyl oxidase was assayed by a modification of the tritium-release assay of Pinnell and Martin (1968). Substrate was prepared using aortae from 7- to 8-day chicks or 18- to 20-day chick embryos as described previously (Myers et al. 1985, Op- sahl et al. 1982). In typical assays, 10 kBq (2–4 mg dry weight) of 3H-labeled substrate were used. Tissue extracts (see below) containing enzyme were added to assay mixtures that contained the aortae substrate suspended in 0.1 mol/L sodium borate buffer containing 0.15 mol/L NaCl, pH 8.0. The samples [usually 100–200 μg as protein] were incubated at 45°C for 2, 4 or 6 h (assay volume 1.5 mL). Assays were performed with substrate alone, with enzyme, or with enzyme preincubated for 30 min in the presence of 50 μg of β-aminopropionitrile per mL of assay buffer. The reactions were stopped by the addition of 0.2 mL of 500 g/L trichloroacetic acid. Released tritium was then determined after centrifugation of individual samples and passage of the supernatant fraction through a column of AG50W-X8(4H) resin (1 × 4 cm). After a wash with two-column volumes of distilled water, an aliquot of the entire eluate was counted for radioactivity by liquid scintillation spectrometry (counting efficiency 30–40%; Myers et al. 1985).

Extraction and quantitation of lysyl oxidase in tissues. For the functional enzyme assays, tissues were homogenized (1–2 min) into 0.01 mol/L sodium phosphate buffer containing 0.15 mol/L NaCl [10:1, v/wt] using a tissue homogenizer at full speed. This homogenate was centrifuged (15,000 × g, 30 min) and the supernatant fraction discarded (see Discussion for rationale). The pellet was then rehomogenized into 0.1 mol/L sodium borate buffer, pH 8.0, containing 4 mol/L urea (10:1, v/wt) and extracted overnight at 4°C. An aliquot of this supernatant fraction (15,000 × g for 30 min) was used for the functional assays after dialysis against or dilution with 0.02 mol/L sodium borate buffer, pH 8.0, to reduce the urea concentration to ≤0.5 mol/L.

For ELISA, tissue samples (500 mg) were transferred to liquid nitrogen and ground to a fine powder. The samples were next extracted into 6 mol/L urea in 0.1 mol/L sodium borate buffer overnight (10:1, v/wt), and appropriately diluted aliquots were taken for ELISA.
addition, lysyl oxidase was estimated by ELISA using supernatant fractions from the guanidinium thiocyanate extractions for RNA isolation (Experiment 2, see below). These samples were dialyzed against 0.1 mol/L sodium borate buffer, pH 8.0, containing 0.5 mol/L urea, the presence of urea at 0.5 mol/L in samples is essential to maintain lysyl oxidase in a disaggregated state (Romero-Chapman 1991). Rat skin, kidney, liver, heart, skeletal muscle (gastrocnemius), smooth muscle (diaphragm), tendon, plasma and aorta from 200 g male Sprague-Dawley rats were used as tissue sources.

**Purification of lysyl oxidase, antibody production, ELISA and Western blotting assays.** Lysyl oxidase, used as a standard, was purified from rat skin as described by Romero-Chapman et al. [1991]. The procedure was modified to include Sephacryl S-200 adsorption as a final step (Shackleton and Hulmes 1990). Lysyl oxidase adsorbs to Sephacryl S-200 in the absence of urea but may be eluted upon addition of buffered 6 mol/L urea. This step, in addition to adsorption onto a column of chick aorta insoluble elastin followed by elution with buffered 6 mol/L urea [see Romero-Chapman et al. 1991], results in rapid isolation of the 32 kDa form of the enzyme. Lysyl oxidase was stored at relatively low concentrations ( aliquots of 20-40 mg/L in 4 mol/L urea at -20°C). Under such conditions, individual preparations were variable stable (2 wk to several months). In contrast, activity is lost completely in 2-3 d when lysyl oxidase was concentrated (>1 g/L) or frozen in the absence of urea.

Immediately after purification the homogenous preparations of lysyl oxidase from rat skin caused the release of 50-100 Bq of tritium per microgram of enzyme, a value nearly identical to those reported by others for highly purified lysyl oxidase (Kagan and Sullivan 1982).

For antibody production, the purified enzyme was concentrated, and a 50-100 µg sample [ loaded in a 5-6 cm trough] was subjected to SDS-PAGE (Burnette 1981, Romero-Chapman et al. 1991). The 32-kDa migrating band of protein was transferred to nitrocellulose. The nitrocellulose to which enzyme was bound was ground to a fine powder and mixed with adjuvant designed for avian antibody production (Ribi Immuno Chemical Research, Hamilton, MO).

The lysyl oxidase adjuvant mixture containing 50 µg of lysyl oxidase was injected into 4-mo-old, white leghorn hens [Avian Science Department, University of California, Davis], followed by weekly booster injections of 10-20 µg of enzyme. Titters of antibodies were observed [10-20 times preimmune serum values] after 4 wk. After 4-6 wk, eggs could also be collected for the extraction and isolation of antilylsyl oxidase antibodies. Procedures for the isolation of chicken immunoglobulins from serum and eggs have been outlined and reviewed by Gassmann et al. [1990]. The antibodies were tested for specificity by Western blotting (Burnette 1981) and their ability to inhibit and absorb lysyl oxidase functional activity extracted from rat skin (see Romero-Chapman et al. 1991 for details).

For ELISA, polystyrene microtiter plates [Dynatech Labs, Alexandria, VA] were coated with antigen. The antilylsyl oxidase (diluted 1:2000) was added, and the reaction with antigen assessed using goat or rabbit antichicken IgG conjugated to horseradish peroxidase (diluted 1:2000). The detection system utilized 3,3,5,5-tetramethylbenzidine and H2O2 as substrates in 0.1 mol/L sodium acetate/citric acid buffer, pH 6 (Romero-Chapman et al. 1991, Tinker et al. 1990).

**cDNA probe construction and Northern assays.** Reverse transcription polymerase chain reaction (RT-PCR) was used to prepare the lysyl oxidase cDNA. RNA (2 µg) from adult rat fibroblasts was used for the reverse transcription (Gacheru et al. 1993). For subsequent PCR amplification, a 10-µl aliquot was removed and adjusted to 100 µL. Antisense primer (CGGTAAATGGTGACGCCAGAGG) and sense primer (GGCACCGACCTGGCATGGGACC) were added at 25 µmol/L, plus 2.5 U Taq polymerase, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 10 µg/mL BSA, and 35 mmol/L each of dATP, dCTP, dGTP, dTTP, buffered with 10 mmol/L Tris-Cl, pH 8.4, buffer. The RNA/DNA hybrid was dissociated at 94°C for 1 min with a ramp time of 1 min. The primers were then annealed (63°C for 30 s), and the sample amplified at 72°C for 120 s (ramp time, 30 s), the cycle was repeated evolve from enteric-derived antigens, because in the chicken the intestine can be a source of antigens that cause nonspecific antibody production. For this assessment, the intestinal contents from white leghorn chickens were extracted into PBS-Tween 20 (1:5 v/v) and the extracted proteins were bound to Affigel-Ald columns as specified by the manufacturer (Sterogen, Arcadia, CA). Normal chicken serum that contained suspected lysyl oxidase binding proteins [as assessed by Western blots] was passed through the Affigel-Ald columns containing the bound enteric-derived protein. Weakly associated components were eluted with PBS-Tween-20 (3 to 4 column volumes); bound components were eluted with 0.1 mol/L borate buffer, pH 8.0, containing 6 mol/L urea. In addition, serum from immunized and nonimmunized chickens [200 µL] was passed through columns of Affigel-Ald to which urea soluble protein from rat skin was bound (1 mg/g gel). Serum proteins that were bound onto the rat skin protein Affigel-Ald columns were eluted as above and also assessed in Western blotting assays. In Western blots, only the protein fraction that absorbed to columns of urea-soluble rat skin protein detected lysyl oxidase in Western blots, i.e., normal chicken serum appears to contain IgG-like proteins that bind to lysyl oxidase. All of the chicken preimmune sera that we tested [6 different lots] demonstrated some level of lysyl oxidase binding capacity in ELISA, although titers were usually one-tenth immune sera resulting from lysyl oxidase and adjuvant injections. Such serum proteins may be important. Lysyl oxidase acts on numerous lysine containing proteins and peptides as substrate. The presence of a serum-binding factor directed at lysyl oxidase may be protective, particularly when lysyl oxidase is released as a feature of extracellular matrix turnover.

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5 During the course of antibody preparation and assessment, we observed that IgG-like proteins are present in normal chicken serum, which bind to lysyl oxidase in Western blots. In a separate experiment, we addressed the possibility that such binding proteins might
an additional 34 times. PCR products were resolved by electrophoresis in 2% agarose and electroeluted. The purified product was eventually cloned into pUC 18 and sequenced (see Svinarchik et al. 1992 for methodological detail) to confirm its identity as rat lysyl oxidase cDNA (Trackman et al. 1990, Trackman et al. 1992). For procollagen (Type I), the cDNA probe was identical to that described by Genovese et al. (1984). The densometric signal relative to that utilizing β-actin cDNA (Gunning et al. 1983) was used as reference. Each probe was randomly primed with 32P-CTP using the multiprime DNA priming system (Amersham, San Francisco, CA). RNA was extracted and isolated using the guanidinium thiocyanate-phenoI-chloroform procedure as described by Chomczynski and Sacchi (1987). To ensure specificity RNA was separated on 1% agarose 1.1 mol/L formaldehyde gels for Northern detection of lysyl oxidase, procollagen α1 chains, and β-actin. Zeta- Probe Hecke GT membranes (Bio-Rad, Richmond, CA) were used for the Northern and slot blot assays. The RNA was fixed onto membranes by transillumination with UV light for 5 min. The membranes were then dried at 80°C (1–2 h). Samples were prehybridized at 65°C for 10 min. For hybridization, the concentration of each probe (labeled with 32P-dCTP) was 1 ng containing approximately 10 Bq/L. Kodak X-OMAT AR film with an intensifying screen (Fisher Scientific, Santa Clara, CA) was used for autoradiography.

**Copper, cross-linking amino acids, soluble collagen, insoluble elastin and tropoelastin assays.** Copper analyses were performed by atomic absorption spectrophotometry (Chung et al. 1988). To estimate the relative solubility of skin collagen, 500 mg of dorsal skin was extracted (4°C) into 1 mol/L NaCl (0.01 mol/L sodium phosphate, pH 7.0) and then into 0.5 mol/L acetic acid (20:1, v/wt, 24 h each). Extracted collagen was estimated as described by Marotta and Martino (1985). The concentration of the reducible cross-linking amino acids, hydroxylysinoonorleucine and dihydroxyornorleucine in the resulting tissue residue after these extractions was also estimated. Data are expressed as moles per mole of collagen α-chain after measurement of hydroxyproline and after reduction of the samples with NABH4[3H] [Reiser et al. 1991].

The levels of extractable tropoelastin (from aorta) and the net deposition of aorta insoluble elastin and its desmosine content (Tinker et al. 1990) provided an additional index of connective tissue protein maturation. Tropoelastin was measured by ELISA as described in Tinker et al. (1990). Aortae were extracted into sodium phosphate buffered (0.05 mol/L, pH 7.6) saline containing 1 g/L Tween 20 for these assays. Insoluble elastin was measured gravimetrically after alkali extraction (0.1 mol/L NaOH, 90°C, 45 min).

**Statistics.** Two sample comparisons employed the Student's t test. Linear regression analyses and estimation of r² were performed as described by Cambell (1989).

### RESULTS

**Estimation of lysyl oxidation by ELISA.** Observations that are important to chicken antilyssyl oxidase serum characterization are given in Figure 1. Western blots utilizing serum from immunized chickens demonstrate its association with the 32-kDA form of lysyl oxidase in rat skin urea extracts (Fig. 1A). The standard curves for the lot of antibody used for determinations herein demonstrate that the assay was sensitive and precise (Fig. 1B). The validation of the ELISA values was based on the excellent correlation between ELISA values and those derived from functional assays (Fig. 1C). Using this assay, the lysyl oxidase concentration of selected tissues and fluids is given in Table 1. Amounts of lysyl oxidase, estimated from assays for functional activity, are also given in Table 1. For those values taken from the literature, values were only used if the assay procedures followed those described by Pinnell and Martin (1968).

The greatest deviation (ELISA vs. amounts derived from functional assays) was for lung, where the estimates of lysyl oxidase from functional assays were lower than would be predicted from corresponding ELISA values. The molar concentrations of lysyl oxidase and corresponding values for tissue copper from literature values or from our laboratory are also given in Table 1. These values indicate that in dense connective tissues, lysyl oxidase can account for 10% or more of the tissue copper.

**Lysyl oxidase protein and functional activity in response to copper deficiency (Experiment 1).** Skin lysyl oxidase activity was significantly low in the skin of rats fed diets containing a low copper diet (2 µg/g of diet) compared with control rats fed a diet containing 10 µg of copper per gram of diet. The lower functional activity, however, was not associated with a significantly lower amount of lysyl oxidase in rat skin estimated by ELISA (Fig. 2). Data used to assess the degree of dietary copper deficiency in this experiment are given in Table 2. Although weight gain was not influenced by the reduction in dietary copper (at 8 wk), plasma and liver copper concentrations were significantly lower in copper-deficient rats compared with control rats.

Further, there were no differences in the relative amounts of skin collagen extracted into a neutral 1 mol/L NaCl and 0.5 mol/L acetic acid solution (Table 2). The cross-link content of residual skin collagen (collagen remaining after 0.5 mol/L acetic acid extraction) was also not affected by the marginal copper sta-
with copper deficiency (Opsahl et al. 1982, Tinker et al. 1990), Table 3.

**Copper deficiency and lysyl oxidase expression (Experiment 2).** To determine if dietary copper influences expression of lysyl oxidase, the levels of lysyl oxidase in different tissues were measured using ELISA and functional assays. The results are summarized in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ELISA estimates[^1] (μg/g)</th>
<th>Functional activity[^2] (kBq/g)</th>
<th>Copper[^3] (μg/g)</th>
<th>Cu in lysyl oxidase [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tendon</td>
<td>571 ± 350 (18 ± 11)</td>
<td>17.5–22.5</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>Skin</td>
<td>158 ± 76 (4.9 ± 2.4)</td>
<td>7.5–10</td>
<td>2.2–2.6 (34–40)*</td>
<td>12–14</td>
</tr>
<tr>
<td>Kidney</td>
<td>154 ± 86 (4.8 ± 2.7)</td>
<td>0.4 ± 0.1**</td>
<td>4.1–6.2 (63–95)**</td>
<td>5–8</td>
</tr>
<tr>
<td>Liver</td>
<td>78 ± 3 (2.4 ± 0.1)</td>
<td>ND</td>
<td>4.1–5.1 (63–78)</td>
<td>3–4</td>
</tr>
<tr>
<td>Lung</td>
<td>72 ± 21 (2.3 ± 0.7)</td>
<td>1.6–3.4*</td>
<td>1.2–1.4 (18–21)**</td>
<td>11–13</td>
</tr>
<tr>
<td>Aorta</td>
<td>32 ± 1 (1.0 ± 0.03)</td>
<td>1.75–2.75*</td>
<td>2.4–2.6 (38–40)*</td>
<td>2–3</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>25 ± 17 (0.8 ± 0.5)</td>
<td>ND</td>
<td>0.8–1.1 (12–17)**</td>
<td>5–7</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>16 ± 5 (0.5 ± 0.2)</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.05 ± 0.002</td>
<td>ND</td>
<td>0.8–0.9 (12–14)</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

[^1]: Values are expressed as μg and nmol (in parentheses) per gram of fresh tissue. The ELISA estimates are the mean of four separate determinations, each done in triplicate, ± 1 SEM. ND indicates not determined.

[^2]: The values are from this laboratory [*] or taken from the literature [**]. The release of 50–100 Bq of tritium in the standard assay represents ~1 μg of functional enzyme. All values represent the range or average amount of lysyl oxidase functional activity assessed for four or more determinations in young adult rats (both the laboratory and literature values). The literature values were obtained for kidney, Chvapil and Misiorowski (1980) and for lung, Almassian et al. (1990), Chichester et al. (1981) and Chvapil and Misiorowski (1980). The values obtained from Chvapil and Misiorowski (1980) were estimated for whole tissue by assuming a protein content of ten percent.

[^3]: Values are from this study [*] or from Keen et al. 1985 [**].

[^4]: Lysyl oxidase (nmol/g tissue) divided by the copper centration (nmol/g tissue) × 100. The calculation assumes one mole of copper per mole of lysyl oxidase (Gacheru et al. 1990).
oxidase, procollagen (Type I α1-chain) and β-actin mRNA transcripts in skin from Cu supplemented and Cu deficient rats were measured [Fig. 3]. Data used to assess the state of copper deficiency (Experiment 2) are given in Figure 3. The data for lysyl oxidase and procollagen are expressed relative to β-actin. The relative levels of Type I procollagen and lysyl oxidase mRNA in skin were not markedly affected by copper status.6

In separate experiments we observed that the levels of lung lysyl oxidase mRNA are often depressed (as much as 70% of normal values) in lung from Cu-deficient rats (<1 μg Cu/g diet). However, the response appears most related to depressed food intake, i.e., the amounts of lysyl oxidase mRNA transcript per unit of β-actin mRNA

**DISCUSSION**

Lysyl oxidase serves a number of essential and diverse functions. Its role in the cross-linking of collagen and elastin is well established. That lysyl oxidase shares >85% identity with rrG protein, a possible antionogene, is another recent and exciting finding [Kenyon et al. 1993]. Therefore, the observation that the level of immunologically detectable protein cor-

| TABLE 2 |
| Indices important to assessment of copper depletion and relative degree of connective tissue maturation in the rats used in Experiment 11 |

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight2</th>
<th>Liver weight2</th>
<th>Liver copper</th>
<th>Plasma copper</th>
<th>Skin collagen solubility3</th>
<th>Collagen cross-links</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>g</td>
<td>μmol/L</td>
<td>μmol/L</td>
<td>mg/g</td>
<td>HLNL4</td>
</tr>
<tr>
<td>Cu-supplemented</td>
<td>192 ± 16</td>
<td>4.95 ± 0.67</td>
<td>64 ± 5</td>
<td>13 ± 3</td>
<td>86 ± 8</td>
<td>0.16</td>
</tr>
<tr>
<td>Cu-deficient</td>
<td>189 ± 27</td>
<td>3.92 ± 0.5</td>
<td>39 ± 14*</td>
<td>4 ± 0.2**</td>
<td>105 ± 21</td>
<td>0.15</td>
</tr>
</tbody>
</table>

1 All values are means [n = 6–12] ± SEM, * P ≤ 0.05, ** P ≤ 0.01, except for HLNL and DHLNL, which are each the average of two determinations.
2 Weight at wk 8 (termination of the experiment).
3 Amount of collagen solubilized into neutral salt (1 mol/L NaCl) and 0.5 mol/L acetic acid (see text for details).
4 Moles of hydroxylysinoenorleucine (HLNL) or dihydroxylysinoenorleucine (DHLNL) per mole of collagen α-chain (insoluble fraction, see text).
TABLE 3
Dietary copper and cross-linking amino acids in aorta elastin isolated from 8 wk-old rats in Experiment 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Aorta&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Insoluble elastin&lt;sup&gt;2&lt;/sup&gt;</th>
<th>DES&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Ide&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Lys&lt;sup&gt;3&lt;/sup&gt;</th>
<th>DES/Ide</th>
<th>(DES + Ide)/Lys</th>
<th>Tropoelastin&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>res/1000 residues</td>
<td>mol/mol</td>
<td>µg/g tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu-supplemented</td>
<td>58 ± 8.5</td>
<td>1.58 ± 0.08</td>
<td>1.21 ± 0.26</td>
<td>0.41 ± 0.09</td>
<td>8.5 ± 4.7</td>
<td>2.94 ± 0.04</td>
<td>0.23 ± 0.05</td>
<td>76 ± 28</td>
</tr>
<tr>
<td>Cu-deficient</td>
<td>40 ± 5.8</td>
<td>1.17 ± 0.06*</td>
<td>1.24 ± 0.24</td>
<td>0.42 ± 0.07</td>
<td>10.4 ± 2.2</td>
<td>2.94 ± 0.09</td>
<td>0.16 ± 0.02</td>
<td>105 ± 48</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM for at least four determinations.
2 Weight of total isolated tissue or weight of total insoluble elastin.
3 Residues of the indicated amino acid per 1000 amino acid residues in the sample.
4 Tropoelastin (as determined in an ELISA) extracted per gram of aorta.

Abbreviations used: Des = desmosine, Ide = Isodesmosine, and Lys = Lysine.
* P < 0.05.

responding to a lysyl oxidase is relatively high in a number of tissues may be of considerable importance (also see Baccarani-Contri et al. 1989, Kagan et al. 1986, Wakasaki and Ooshima 1990). In dense connective tissue, such as tendon and skin, where lysyl oxidase is concentrated (>150 µg/g tissue), the amount of lysyl oxidase is more than sufficient to accommodate the normal cross-linking of collagen. For example, one nanomole or ~32 µg lysyl oxidase can theoretically produce 300–400 nmol of lysyl-derived aldehydic functions, based on the enzyme’s ability to carry out at least 300–400 catalytic cycles in typical assays. Theoretically, this is more than enough enzyme to effect the cross-linking of 100 nmol or 90 mg of fibrillar collagen, assuming 3–4 cross-links per α-chain (see Romero-Chapman et al. 1991, Trackman et al. 1981). Because the turnover of collagen in mature skin and tendon is best estimated as weeks (Reiser et al. 1992), it is inter-

**FIGURE 3** Copper status and steady-state levels of messenger RNA for lysyl oxidase and procollagen α<sub>1</sub> relative to β-actin (Experiment 2) in rat skin. Values are means ± SEM for two to three determinations. Rats were fed copper-deficient (<0.5 µg/g or copper-supplemented [10 µg/g diets from weaning for 21 d. At termination, values for body weight, liver copper concentration, and hematocrit were as follows: 128 ± 7 and 118 ± 10 g, 5 ± 3 and 0.67 ± 0.4 nmol Cu/g, and 0.46 ± 0.002 and 0.38 ± 0.001 for copper-supplemented and copper-deficient rats, respectively. The levels of mRNA for lysyl oxidase and procollagen α<sub>1</sub> are expressed relative to β-actin. The cDNA probes used for the estimates were assessed in Northern assays. Procollagen mRNA was expressed as 5.7 and 4.7 kb transcripts. The primary transcript for lysyl oxidase was 4.5 kb with a minor signal at 1.8–2.0 kb. β-Actin was observed as a 2.1 kb transcript. For quantitation, slot blot analyses were performed. Slot blots were exposed to Kodak X-OMAT for AR film: lysyl oxidase [7 d], procollagen α<sub>1</sub> [2–3 d] and β-actin [1 d].
testing that lysyl oxidase levels are substantial and are easily measured in adult animals (Reiser et al. 1992).

It is also clear that for some organs, particularly at specific periods in organogenesis, e.g., formation of the aorta or the transition from a saccular to alveolar lung, any impairment in lysyl oxidase activity is critical (Dubick et al. 1985, Reiser et al. 1992). In this regard, using chemical or biomechanical criteria, others have also observed that the maturation of collagen in skin and tendon is not as responsive to copper deficiency as maturation of collagen in aorta or lung (see Reiser et al. 1992 and references cited). For example, Chou et al. (1968) reported that in severely copper deficient chicks, the solubility of skin collagen is the same as that for copper-supplemented chicks, although a fivefold difference was observed in aorta collagen solubility (copper-deficient vs. supplemented groups). The 3- to 10-fold differences in concentrations of lysyl oxidase in various tissues may provide an explanation. The inability to observe changes in skin collagen cross-linking (Table 2), in contrast with changes reported for aorta and lung elastin and collagen crosslinking (see Dubick et al. 1985, Reiser et al. 1992), may be due to the relatively lower tissue concentration of lysyl oxidase in aorta and lung.

Kagan and co-workers (Kagan and Sullivan 1982, Trackman et al. 1981) observed that preparations of purified lysyl oxidase cause the release of 50–100 Bq of tritium per microgram of enzyme when assayed by the method of Pinnell and Martin (1968). Likewise, we observed that within the operational limits of the Pinnell and Martin assay, tritium release can be used as an index to assess relative amounts of enzyme. As an important extension of this observation, a survey of the literature on lysyl oxidase in the rat (Table 1) and other species [chickens, humans, mice] (Table 4) leads to the conclusion that dense connective tissue contains substantial quantities of lysyl oxidase.

For tissues wherein lysyl oxidase is difficult to measure consistently in functional assays, there are several possible explanations. In addition to differences in expression or turnover, the presence of lysyl oxidase inhibitors has been emphasized by Narayanan et al. (1982), who observed inhibition of lysyl oxidase activity by components in neutral salt extracts of tissues and serum. The values for lysyl oxidase functional activity often increase several-fold when tissue samples are extracted briefly with buffered saline (to remove inhibitors) before extraction into buffered solutions of urea to solubilize enzyme for functional assay. This may be the case for kidney, which was characterized by a high concentration of lysyl oxidase protein, i.e., as measured by ELISA, but low functional activity. Lysyl oxidase may also serve a structural role in the extracellular matrix (Kagan et al. 1986).

Another important feature of these data is related to copper. Given that one mole of copper is associated with one mole of lysyl oxidase (Gacheru et al. 1990), the observation that lysyl oxidase in many instances accounts for 10% or more of tissue copper suggests that factors that influence lysyl oxidase may also be a consideration in the overall metabolism of tissue copper. In the rat, the apparent half-life of copper in skin is 50–70 h (Romero-Chapman et al. 1991). Although no precise estimates for lysyl oxidase turnover exist, the suggestion can be made that lysyl oxidase may be a quantitatively important vehicle for the export of copper for some connective tissue cells.

In summary, lysyl oxidase is quantitatively a major enzyme in connective tissues. In addition to being influenced by copper status, lysyl oxidase may also be

<table>
<thead>
<tr>
<th>Species</th>
<th>Source or tissue</th>
<th>Functional activity</th>
<th>Estimated amount</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Fibroblasts</td>
<td>0.3–0.45/10^6 cells</td>
<td>~4/10^6 cells</td>
<td>Gacheru et al. 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.35–0.45/10^6 cells</td>
<td>~4/10^6 cells</td>
<td>Kuivaniemi et al. 1984</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>&lt;2.5/L</td>
<td>~6/g tissue</td>
<td>Murawski et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Placenta</td>
<td>0.5–0.6/g tissue</td>
<td>~300/g tissue</td>
<td>Kuivaniemi et al. 1984</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>25–30/g</td>
<td>~50/g tissue</td>
<td>Peltonen et al. 1983</td>
</tr>
<tr>
<td>Chicken</td>
<td>Aorta</td>
<td>~5/g tissue</td>
<td>~50/g tissue</td>
<td>Rayton and Harris 1979</td>
</tr>
<tr>
<td></td>
<td>(1–3 wk old)</td>
<td>3.8–7.0/g tissue</td>
<td>~50/g tissue</td>
<td>Harris et al. 1974</td>
</tr>
<tr>
<td></td>
<td>Cartilage [3-wk old]</td>
<td>2/g tissue</td>
<td>~20/g tissue</td>
<td>Rucker and Goettlich-Riemann 1972</td>
</tr>
<tr>
<td></td>
<td>Cartilage [17-d embryo]</td>
<td>0.125/mg soluble protein</td>
<td>~1.0/mg soluble protein</td>
<td>Stassen 1976</td>
</tr>
<tr>
<td>Mouse</td>
<td>Uterine cervix</td>
<td>0.005/mg soluble protein</td>
<td>~6/10^6 cells</td>
<td>Ozasa et al. 1981</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>0.3–0.6/10^6 cells</td>
<td>~6/10^6 cells</td>
<td>Peltonen et al. 1983</td>
</tr>
<tr>
<td>Bovine</td>
<td>Aorta</td>
<td>1–1.25/g</td>
<td>~12/g tissue</td>
<td>Burbelo et al. 1985</td>
</tr>
</tbody>
</table>

1 Based on the observation that 1 μg of functional enzyme from various sources causes the release of 50–100 Bq of [3H]H_2O in the Pinnell and Martin assay (1968). Fibroblasts at 10^6 cells constituted ~ 0.05–0.1 mL of packed cells.
important in the normal export of copper from connective tissue cells.

LITERATURE CITED


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