Aortic tissue from chicks raised 8 to 11 days on diets lacking copper contains diminished levels of the enzyme lysyl oxidase, a copper metalloenzyme. By incubating the deficient aortic tissue in fully oxygenated Waymouth growth medium (MB 751/2) supplemented with 3 to 5 μg/ml of CuSO₄, it was possible to restore lysyl oxidase activity to the aorta. Enzyme activation required copper supplements to the serum-free medium. When a partially purified preparation of serum copper proteins supplied the copper, activation was also achieved but at a much lower copper concentration (0.2 μg/ml). Homogenizing the tissue or incubating it under N₂ or in the cold blocked the appearance of the copper-induced enzyme activity. Incubating the tissue in a simple solution containing buffer salts and CuSO₄ did not result in activation. A time course analysis showed that 3- to 5-h lag period preceded the appearance of enzyme activity in the tissue. Further studies with [³H]lysine and "Cu revealed that the metal became bound to a newly synthesized protein component which affinity to a collagen-derivatized Sepharose 4B column and eluted with 6 M urea, typical of the behavior of lysyl oxidase. On polyacrylamide gel in the presence of sodium dodecyl sulfate, this component migrated with a molecular weight of about 60,000, approximately the molecular weight of chick aorta lysyl oxidase. Cycloheximide, but not actinomycin D, completely inhibited the incorporation of both [³H]lysine and "Cu into the 60,000 molecular weight component. These data obtained with aortic tissue in a defined medium suggest that activation of lysyl oxidase with copper is a property of metabolically active tissues and proceeds with the binding of copper to newly synthesized protein. The findings strongly support the possibility that induction involves enzyme synthesis rather than enzyme activation.

The induction of metalloproteins by metal ions has been observed in a wide range of animal tissues and microbial organisms. The prototype for such studies has been the protein ferritin, a major iron storage protein in liver and other organs (1, 2). Since these earlier studies, numerous reports have appeared describing the regulation of nonferrous metalloproteins and metalloenzymes by specific metal ions (3-10). For example, daily injections of copper salts administered to weaned rats markedly stimulated the biosynthesis of ceruloplasmin, the major copper-binding protein in serum (3). Similarly, intraperitoneal injections of cadmium salts increased the intracellular concentrations of a cadmium-binding protein in kidney and liver (4, 5). Intracellular zinc (6) and copper (7, 8) binding proteins, which are normally present in very low amounts, are elevated sharply by increases in the specific intracellular metal ion concentration. Furthermore, such stimulated increases in metalloproteins are usually abolished by actinomycin D and cycloheximide, supporting the contention that the metal is actually inducing their synthesis (5, 6, 10). If metal ions can induce the synthesis of metalloproteins, it is imperative to know the biochemical mechanism.

Since the majority of metalloproteins discovered thus far function as enzymes, a pertinent question is whether metalloenzyme activity is under inductive control by metals. Metalloenzymes have limited and quite specific binding capacities for metals and thus may have regulatory mechanisms different from those of metalloproteins. The question also hints at the possibility that the specific metal ion component of a metalloenzyme must be present to permit the synthesis of the protein moiety. Current evidence suggests that for some metalloenzymes this may not be the case. For instance, Keyhani et al. (12) reported that yeast cells grown in a medium deficient in copper accumulate proportionate numbers of the subunit components of cytochrome oxidase. Final assembly into a functioning enzyme, however, requires the presence of copper. Likewise, rats exposed to a moderate copper deficiency continue to synthesize and release a serum protein which resembles ceruloplasmin immunochemically but contains very little detectable copper (13). Such results suggest that the synthesis of apo ceruloplasmin or cytochrome oxidase does not depend upon the presence of copper. They further suggest that insertion of copper into these metalloenzymes occurs after translation.

Lysyl oxidase is a copper metalloenzyme found primarily in connective tissue. The enzyme, a specific amine oxidase, catalyzes the oxidative deamination of peptidyl lysine residues in collagen and elastin preparatory to the formation of covalent cross-links in these proteins (14). Lysyl oxidase has been purified to homogeneity by different purification schemes (15-17). Chick aorta lysyl oxidase has a molecular weight of about 60,000 and like its counterpart in bovine aorta (18) shows evidence for multiple molecular forms (19), perhaps accounting for known differences in immunochemical (20) and inhibitory properties of lysyl oxidase (21). Low levels of dietary copper lower the lysyl oxidase activity in chick aorta (15) and

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§ To whom all correspondence concerning this manuscript should be sent.

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1 No Enzyme Commission (EC) number has been assigned to this enzyme.
cartilage (22), clearly demonstrating the importance of copper to enzyme function. However, enzyme activity is restored by injecting trace amounts of CuSO₄ (less than 0.5 pmol) intraperitoneally into deficient chicks (23). The activation process is blocked by cycloheximide but not actinomycin D, suggesting that copper somehow affects a post-transcriptional regulatory process in aortic tissue. Further probes of the activation mechanism have led to the present work. Here we report that lysyl oxidase activity can be restored to deficient aortas in vitro when the largely intact tissues are incubated in a serum-free Waymouth medium. The copper concentration in the medium appears to control the response. A preliminary account of this work has appeared (24).

MATERIALS AND METHODS

Animals—Male, inbred, hybrid white leghorn chicks were obtained on the day of hatching and housed in all plastic (Plexiglas) cages (51 × 76 × 35 cm), maintained at 28-32°C. Chicks were fed deionized distilled water and semipurified diets ad libitum prepared as described by O'Dell et al. (25). Diets of deficient animals contained by analysis (atomic absorption spectrophotometry) less than 1 µg of copper/g of diet. Control diets were identical in contents but were supplemented with 50 µg of anhydrous CuSO₄/g of diet.

Preparation of Aortic Tissues for Cultures—Chicks were killed after 8 to 14h of age. Hearts and aortas were placed in Waymouth MB 751/2 growth medium in a Petri dish kept at room temperature. Aortas from deficient chicks were quite fragile and care was taken not to tear the tissue during isolation.

Incubation of Aortas—Aortic tissue was transferred to screw-capped culture tubes (15 × 0.8 mm) (two aortas/tube) with 2.0 ml of fresh Waymouth medium (26). Penicillin G (50 units/ml) and streptomycin sulfate (50 µg/ml) were added to suppress bacterial contamination. Additional components to the medium are described in the tables and figure legends. Culture tubes were gassed with 100% oxygen for 5 s, sealed, then incubated at 37°C in a shaking water bath.

Preparation of Aortic Extracts—All operations were performed at 0-4°C. Incubated aortas in pairs were removed from the tube with a spatula, blotted dry, and weighed. The tissue was homogenized (Teflon on glass) in a solution of 0.12 M NaCl, 0.015 M Na₂HPO₄, pH 7.6 (Buffer A) in a final suspension of 5% (v/v). The homogenate was centrifuged (10,000 × g; 10 min) and the supernatant was discarded. The pellet fraction was resuspended (5%, w/v) with homogenization in 6 M urea in Buffer A, pH 8.3, and placed in an ice bath for 2 h. After centrifugation (105,000 × g, 30 min), the clear supernatant was dialyzed against 200 volumes of Buffer A changed twice over 20 h and the dialyzed extract was tested for enzyme activity. Less than 7% of the lysyl oxidase activity in chick aorta is extractable with salt solution and consequently the intact tissue was incubated in Waymouth MB 751/2 growth medium in a Petri dish kept at room temperature. Aortas from deficient chicks were quite fragile and care was taken not to tear the tissue during isolation.

Incubation of Aortas—Aortic tissue was transferred to screw-capped culture tubes (15 × 0.8 mm) (two aortas/tube) with 2.0 ml of fresh Waymouth medium (26). Penicillin G (50 units/ml) and streptomycin sulfate (50 µg/ml) were added to suppress bacterial contamination. Additional components to the medium are described in the tables and figure legends. Culture tubes were gassed with 100% oxygen for 5 s, sealed, then incubated at 37°C in a shaking water bath.

Acrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate—The method of Laemmli was followed (28). Duplicate gels were run for each sample in a Hoefer model PE 12 apparatus. Exactly 0.2 ml of the concentrated protein solution was diluted 1:1 in a buffer made up by mixing 19 ml of 0.625 M Tris-HCl, pH 6.8, with 10 ml of 10% (w/v) sodium dodecyl sulfate and adding 1.0 ml of 2-mercaptoethanol, 27 ml of glycerol, and 1 mg of bromphenol blue. Samples in this buffer were heated for 2 min in a boiling water bath. After the electrophoresis run, one gel was stained with 25% (v/v) Coomasie brilliant blue (dissolved in an aqueous solution of 25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid) for 30 min and destained by diffusion in 7.5% (v/v) acetic acid. The other was cut into 2-mm sections which were placed in scintillation vials and immersed in exactly 0.2 ml of 30% (v/v) H₂O₂. After the gels had dissolved (12 h at 40°C), 6 ml of Aquasol was added to each vial and both H and Cu were quantitated in the Beckman counter.

RESULTS

Requirement for Copper—The aim of the experiments was to investigate the feasibility of activating aortic lysyl oxidase in vitro with copper. The simplest approach was to take aortas from 8- to 11-day copper-deficient chicks and transfer the largely intact tissue to Waymouth's growth medium. Aortas so treated when suspended in the medium for 18 h failed to show an increase in lysyl oxidase activity. Adding serum from copper-fed chicks to the medium also produced no response. However, when CuSO₄ was added to a final concentration of 5 µg of copper/ml, substantial amounts of enzyme activity appeared in the tissue after 18 h (Table I). The serum-free medium contained about 0.1 µg of copper/ml present as a contaminant from the other chemicals. This amount of copper apparently could not support the activation. Note, however, that raising the copper concentration from 0.1 µg/ml to 1.0 µg/ml brought about a g-fold increase in enzyme activity in the tissue (Fig. 1). Such copper-induced activation was maximum at 5.0 µg of copper/ml. Clearly the response was dependent on the medium copper concentrations. When ZnSO₄ and FeSO₄ were tested separately at the same effective metal ion concentration (5 µg/ml), these salts did not achieve the same degree of activation as did CuSO₄ (Table II).

Activation with Protein-bound Copper—The level of copper needed to achieve maximum activation was 20 to 25 times

In those experiments in which Cu uptake was determined, the supernatant was retained for analysis.

We thank Dr. R. C. Siegel for generously supplying the rat tail collagen.

Assay of Lysyl Oxidase Activity—The procedure followed that of Pinnell and Martin (27) with modifications as noted earlier (23). The protein substrates were prepared from 6-day-old chick aortas incubated for 24 h in a lysine-free Waymouth medium supplemented with 20 µCi of L-[4,5-³H]lysine (Schwarz/Mann, specific activity 35 mCi/mg) and 25 µg/ml of bovine urine lysozyme (lysazyme). Approximately 500,000 cpm of substrate proteins were mixed with 0.2 ml of extract containing the enzyme. Buffer A was added to a final volume of 1 ml. Incubations lasted for 2 h at 40°C. Exchangeable tritium (as tritiated water) was separated by vacuum distillation and 0.2 ml of the distilate in 5.0 ml of Aquasol scintillation fluid was measured for radioactivity in a Beckman model 200 liquid scintillation counter, with a counting efficiency of 30% for tritium. The enzyme activity is expressed in counts per min of tritiated water formed per hour per wet weight of tissue. The enzyme assays typically show a linear release of H₂O for at least 4 h.

Measurement of H and Cu in Protein Samples—Radioactive copper (¹³Cu, 3.0 mCi/mg) was purchased from New England Nuclear Inc. Measurements of ¹³Cu and H incorporation into aortic proteins were conducted after purifying the proteins on affinity columns (see above). About 3.5 ml of the column eluate in 6 µl urea buffer were dialyzed against 1000 volumes of deionized distilled water containing 10 µM cuprizone to remove loosely bound copper (two times over 6 h) before transfer of the eluate in water wash on the chromatofocusing column. Five-tenths milliliter of this was mixed directly with 5 ml of Aquasol and measured for H and ¹³Cu in the Beckman counter. Samples were counted twice: once for H and ¹³Cu and, then, after sitting in the dark for 1 week to allow ¹³Cu to decay to background levels, a second time for H alone. The remaining 3.0 ml from each of the four replicates were pooled and concentrated five times by vacuum distillation at room temperature.

Two-tenths milliliter of the concentrated protein solution was further analyzed by the electrophoresis procedure described below.

Activation with Protein-bound Copper—The aim of the experiments was to investigate the feasibility of activating aortic lysyl oxidase in vitro with copper. The simplest approach was to take aortas from 8- to 11-day copper-deficient chicks and transfer the largely intact tissue to Waymouth's growth medium. Aortas so treated when suspended in the medium for 18 h failed to show an increase in lysyl oxidase activity. Adding serum from copper-fed chicks to the medium also produced no response. However, when CuSO₄ was added to a final concentration of 5 µg of copper/ml, substantial amounts of enzyme activity appeared in the tissue after 18 h (Table I). The serum-free medium contained about 0.1 µg of copper/ml present as a contaminant from the other chemicals. This amount of copper apparently could not support the activation. Note, however, that raising the copper concentration from 0.1 µg/ml to 1.0 µg/ml brought about a 6-fold increase in enzyme activity in the tissue (Fig. 1). Such copper-induced activation was maximum at 5.0 µg of copper/ml. Clearly the response was dependent on the medium copper concentrations. When ZnSO₄ and FeSO₄ were tested separately at the same effective metal ion concentration (5 µg/ml), these salts did not achieve the same degree of activation as did CuSO₄ (Table II).
TABLE I
Serum and copper requirements for lysyl oxidase activation in vitro

<table>
<thead>
<tr>
<th>Nutritional status of chicks</th>
<th>Additions to Waymouth medium</th>
<th>Lysyl oxidase activity (×10^-6)</th>
<th>cpm H2O/h/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper-fed</td>
<td>Serum (Cu⁺)</td>
<td>35.7 ± 6.1</td>
<td>8 ± 1.7</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>Serum (Cu⁺)</td>
<td>64 ± 2.2</td>
<td>6 ± 1.7</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>CuSO4</td>
<td>24.4 ± 4.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>Serum (Cu⁺) + CuSO4</td>
<td>20.7 ± 2.2</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>Serum (Cu⁺) + CuSO4</td>
<td>21.4 ± 3.1</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

* Source of serum was either deficient (Cu⁻) or control (Cu⁺) chicks.

TABLE II
Effects of Cu²⁺, Zn²⁺, and Fe³⁺ salts on the in vitro activation of aortic lysyl oxidase

<table>
<thead>
<tr>
<th>Salt added</th>
<th>Lysyl oxidase activity (×10^-6)</th>
<th>cpm H2O/h/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.82 ± 1.7</td>
<td>8.33 ± 7.91</td>
</tr>
<tr>
<td>CuSO4</td>
<td>23.70 ± 1.32</td>
<td>8.33 ± 7.91</td>
</tr>
<tr>
<td>FeSO4</td>
<td>11.76 ± 5.72</td>
<td>8.33 ± 7.91</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>11.76 ± 5.72</td>
<td>8.33 ± 7.91</td>
</tr>
</tbody>
</table>

TABLE III
The in vitro activation of aortic lysyl oxidase by serum copper proteins

<table>
<thead>
<tr>
<th>Agent added</th>
<th>Copper level</th>
<th>Protein level</th>
<th>Lysyl oxidase activity (×10^-6)</th>
<th>cpm H2O/h/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>0.2</td>
<td>1.0</td>
<td>4.2 ± 0.48</td>
<td>4.2 ± 0.48</td>
</tr>
<tr>
<td>Copper-binding proteins</td>
<td>0.2</td>
<td>1.0</td>
<td>14.6 ± 1.02</td>
<td>14.6 ± 1.02</td>
</tr>
<tr>
<td>Copper</td>
<td>0.2</td>
<td>1.0</td>
<td>7.9 ± 0.51</td>
<td>7.9 ± 0.51</td>
</tr>
<tr>
<td>CuSO4</td>
<td>0.2</td>
<td>1.0</td>
<td>6.8 ± 0.84</td>
<td>6.8 ± 0.84</td>
</tr>
<tr>
<td>CuSO4</td>
<td>5.0</td>
<td>1.0</td>
<td>21.4 ± 1.39</td>
<td>21.4 ± 1.39</td>
</tr>
</tbody>
</table>

higher than the concentration of copper in normal chick blood which is about 0.2 µg/ml (29). As to why the in vitro system required such high amounts of copper was not determined directly. It did suggest, however, that aqueous complexes of copper were not the physiological form of the metal, even though such complexes apparently could force the activation of the enzyme in vitro. Thus, it was important to determine whether the in vitro system could respond to a protein-bound copper at physiological levels of the metal. To achieve the objective, the copper-binding proteins in chick serum were isolated by chromatographic procedures employing Sephadex G-100 and DEAE-cellulose. Fractions which contained nondialyzable copper were pooled and concentrated by lyophilization. Based on the copper/protein ratios, such copper proteins had an estimated purity of about 50-fold over those present in normal serum. When these proteins were resuspended in the Waymouth medium so as to contribute a copper concentration of 0.2 µg/ml, substantial activation of the aortic lysyl oxidase was observed after 12 h (Table III). An albumin-copper complex, prepared by exposing bovine serum albumin to excess CuSO4 followed by dialysis of the complex to remove unbound metal, also produced activation, but the amount was quite low. In fact, the albumin-bound copper was as effective as the free metal at the same concentration. Higher concentrations of protein-free copper, however, produced the strongest activation, confirming the earlier results. The serum copper proteins were tested at only one concentration so it is not known whether higher protein concentrations would elicit more activity. It seems clear, however, that the induced response can be achieved with much lower copper concentrations but at these lower concentrations a specific copper protein is needed.

Kinetic Studies—Kinetic studies were performed to 1) investigate the time of optimum activation and 2) the rapidity with which enzyme activity appeared after copper was added to the incubation medium. A close kinetic analysis showed that the copper-induced activation in vitro did not occur immediately but rather followed a delay period of about 3 to 5 h (Fig. 2). With no copper supplements there was no rise in enzyme activity at any time over a 25-h period. The delay period which characterizes the response in vitro is reminiscent of the activation of lysyl oxidase in the intact chick. There, a 1- to 2-h delay period after copper injections was typical and was assumed to be caused by the slow absorption and transport of copper to the aorta. That supposition may no longer be correct.

Other Properties of the System—A summary of other important variables which were observed to play a role in the activation of lysyl oxidase in the in vitro system is shown in Table IV. Briefly, disrupting the tissue by homogenization, incubating it under N2 or at 3°C, or substituting buffer salts for the Waymouth medium were treatments which abolished or lessened the copper-induced activation. The results suggest that activation of the enzyme in this manner is an energy-dependent process requiring intact, structurally sound tissue. They tend to eliminate the possibility that the activation is simply a fortuitous interaction of aqueous copper with the metal-free enzyme.

Studies of the Activation Mechanism—With the attainment of a simple reproducible system above, studies were
undertaken to investigate the molecular events that lead to activation. Aortic tissues from deficient and control (copper-fed) were incubated in Waymouth’s medium supplemented with CuSO₄ and 2.6 μCi/ml of Cu. After 12 h, the tissue was washed with a buffer containing 0.1 mM cuprozine to chelate loosely bound copper. The dialyzed extracts were further purified by passage through a collagen-derivatized Sepharose column which bound the enzyme (see “Materials and Methods”). Elution of the enzyme from the column yielded a more purified but apparently nonhomogenous prep with a recovery

**TABLE IV**

Conditions for activating aortic lysyl oxidase in vitro with copper

All aortas were obtained from 8-day deficient animals. The aortas were incubated for 12 h under the conditions shown. Those which were ground were homogenized under sterile conditions in the incubation medium until the structural state of the aorta was destroyed. Tubes were gassed with either O₂ or N₂ before sealing. Data ± S.E. n = 3.

<table>
<thead>
<tr>
<th>Waymouth medium</th>
<th>Added copper (μg/ml)</th>
<th>Buffer salts</th>
<th>Temperature</th>
<th>State of tissue</th>
<th>Surrounding gas</th>
<th>Lysyl oxidase activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10⁻⁷</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>37</td>
<td>Intact</td>
<td>100% O₂</td>
<td>4.25 ± 0.85</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>37</td>
<td>Intact</td>
<td>100% O₂</td>
<td>17.47 ± 3.08</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>37</td>
<td>Intact</td>
<td>100% N₂</td>
<td>8.46 ± 0.79</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>37</td>
<td>Ground</td>
<td>100% O₂</td>
<td>9.91 ± 1.86</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>37</td>
<td>Ground</td>
<td>100% N₂</td>
<td>10.04 ± 1.99</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>37</td>
<td>Intact</td>
<td>100% N₂</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>−</td>
<td>37</td>
<td>Intact</td>
<td>100% N₂</td>
<td>11.09 ± 1.3</td>
</tr>
</tbody>
</table>

*contained 2.1 mM Na₂HPO₄, 0.6 mM KH₂PO₄, and 26.7 mM NaHCO₃, pH 7.4.

**TABLE V**

Incorporation of [³H]lysine and [⁶⁴Cu] into aortic proteins: effect of cycloheximide and actinomycin on incorporation and lysyl oxidase activation

After the incubation, dialyzed extracts containing the enzyme were passed through affinity columns as a means to further purify the enzyme. Data ± S.E. n = 4.

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>Copper added (μg/ml)</th>
<th>Antimetabolite</th>
<th>[³H]lysine incorporated (cpm)</th>
<th>[⁶⁴Cu] incorporated (cpm)</th>
<th>Lysyl oxidase activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10⁻⁴</td>
<td>10⁻⁸</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>Copper-fed</td>
<td>+</td>
<td>None</td>
<td>64.20 ± 6.5</td>
<td>67.88 ± 4.8</td>
<td>2.30 ± 0.7</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>+</td>
<td>None</td>
<td>50.20 ± 3.8</td>
<td>74.75 ± 4.8</td>
<td>4.02 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>None</td>
<td>98.40 ± 6.3</td>
<td>11.09 ± 1.3</td>
<td>1.06 ± 0.2</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>+</td>
<td>Cycloheximide</td>
<td>1.44 ± 0.0</td>
<td>2.08 ± 0.0</td>
<td>4.22 ± 1.1</td>
</tr>
<tr>
<td>Copper-deficient</td>
<td>+</td>
<td>Actinomycin D</td>
<td>49.2 ± 2.9</td>
<td>77.44 ± 7.9</td>
<td>4.82 ± 1.1</td>
</tr>
</tbody>
</table>

*Corrected for disintegrations apparent at zero time. The actual counts observed were between 240 and 465 cpm.

b 10 μg/ml.

c 25 μg/ml.
Activation of aortic lysyl oxidase in vitro following treatment with $\beta$-aminopropionitrile fumarate

Aortic tissue was obtained from 6-day-old copper-fed chicks. The first incubation was in Waymouth medium (60 min, 37°C) containing 50 $\mu$g/ml of $\beta$-aminopropionitrile fumarate. The tissue was transferred to fresh medium containing supplements as shown (cycloheximide, 10 $\mu$g/ml; CuSO$_4$, 5 $\mu$g of copper/ml) and incubated at 37°C for 18 h. Data ± S.E. n = 5.

<table>
<thead>
<tr>
<th>First incubation</th>
<th>Second incubation</th>
<th>Lysyl oxidase activity ($\times 10^4$ cpm/$\text{H}_2\text{O}/h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO$_4$</td>
<td>No additions</td>
<td>23.6 ± 1.3</td>
</tr>
<tr>
<td>BAPN</td>
<td>No additions</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>BAPN</td>
<td>Cycloheximide</td>
<td>11.5 ± 0.6</td>
</tr>
<tr>
<td>BAPN</td>
<td>CuSO$_4$</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>BAPN</td>
<td>CuSO$_4$ + cycloheximide</td>
<td>11.6 ± 0.6</td>
</tr>
</tbody>
</table>

*a* No incubation.

DISCUSSION

Copper exerts close control over lysyl oxidase, a copper metalloenzyme in aortic tissue. The phenomenon, observed first in vivo (23), has now been observed in intact isolated aortic tissue suspended in a chemically defined medium. The activation of lysyl oxidase with copper is clearly dependent on the concentration of copper added to the medium, high concentrations of the metal ions appearing capable of overcoming a need for serum proteins. However, the isolated tissue showed partial activation when homologous serum copper proteins were added, suggesting that the simple in vitro system was capable of reproducing events which occurred physiologically.

The apparent requirement for a fully supplemented growth medium is a significant feature. The growth medium supplies amino acids, some of which are known to bind copper and hasten its uptake into suspended liver slices (32). However, enzyme activation was never immediate in the in vitro system; a 3- to 5-h delay was typical and reminiscent of copper-induced activation in vivo (23). Since low temperatures, homogenization, and anaerobic conditions diminished the activation response, it seems clear that activation of lysyl oxidase in vitro is a property of intact metabolically active tissue. The Waymouth growth medium, unlike simple buffer salts, supplies those ingredients which sustain growth as well as chelate copper. Moreover, the sensitivity to very low concentrations of cycloheximide suggests that full activation occurs only when protein synthesis is uninterrupted. The fact that cycloheximide did not completely shut off lysyl oxidase activation by copper could be due to the reversible nature of cycloheximide inhibition (30). Such activity could reflect protein synthesis delayed in the incubation period when inhibitory effects had been overcome.

In the present study, the copper-induced activation was accompanied by the binding of radioactive copper to a 60,000-dalton protein which attached to collagen-derivatized Sepharose. The protein-copper complex was of sufficient stability to permit analysis on electrophoresis gels in the presence of detergents. As yet, it is not known how many copper atoms became bound to 1 molecule of the enzyme. However, when protein synthesis was blocked with cycloheximide, no radioactive copper became bound to protein. Thus, a diminution in activation correlated with the diminished binding of $^{64}$Cu to protein and both events showed a dependence on de novo synthesis of protein. Since the identity of the 60,000-dalton component has not been established conclusively, there is the possibility that this component is an activator or transport protein, or both, whose synthesis is a key event in the activation mechanism. The procedure for preparing the protein makes this possibility remote.

That copper did not bind to any protein when protein synthesis was blocked strongly suggests that the insertion of the metal into the protein structure must occur at translation or before the newly assembled protein has equilibrated with its intracellular pool. Failure to bind with inhibitor present could also mean that apoenzyme components are not present or, if so, are incapable of binding copper. The data do not permit a distinction. Nonetheless the lysyl oxidase system appears to contrast with that of cytochrome oxidase in yeast. There copper becomes incorporated into preassembled subunits which accumulate although sufficient supplies of copper.

| Data + SE. n = 5. |

*The abbreviation used is: BAPN, $\beta$-aminopropionitrile fumarate.
are not available (12). If copper incorporation into lysyl oxidase is synchronized with specific stages of protein assembly, this could explain why activation cannot be achieved in homogenized tissues (Table IV) or in extracts from copper-deficient aortas.6 The findings in fact have relevance to the observation that the protein moiety of ceruloplasmin, once synthesized, will not bind nor exchange serum copper atoms (33) unless its structure is modified chemically (34).

In the rapidly growing chick, aortic lysyl oxidase has an estimated half-life of about 16 h (35). Thus, lysyl oxidase does not escape the usual turnover processes that control protein levels. Assuming minimum recycling of copper, the stress of copper from serum proteins to the metal-free enzyme, thus overcoming some of these difficulties and hopefully eventually activates lysyl oxidase with copper occurs via pathways yet to be identified and a mechanism largely unknown. Although intracellular copper-binding proteins have been identified in many tissues (8, 10, 36-40), as yet no one has reported their existence.

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**REFERENCES**


*6 F. D. Harris and J. K. Rayton, unpublished results.*