Cytochrome c Oxidase

THE BATHOCUPROINE EFFECT*

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SUMMARY

When a purified bovine heart cytochrome c oxidase was polarographically assayed by a method involving initial contact of the enzyme with Tween 80, very high turnover numbers were observed at low enzyme concentrations, e.g. 5 to 10 μM cytochrome c. When substrate-reduced enzyme was treated with bathocuproine, the high turnover numbers at low enzyme concentrations were abolished but could be recovered by further treatment of the inhibited enzyme with Cu²⁺. This new form of inhibition of the enzyme, called the "bathocuproine effect," is ascribed to an interference by the reagent of enzyme dissociation to a unit aggregate of maximal activity. The effect is not due to copper depletion of cytochrome c oxidase.

2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonate (bathocuproine), a chelator of Cu²⁺, is not an inhibitor of cytochrome c oxidase (1, 2). Nevertheless, we have found that when the oxidase, reduced by substrate, is treated with this reagent in the presence of a surface-active agent, the characteristic relationship between concentration and turnover number is profoundly modified. This "bathocuproine effect" can be reversed by Cu²⁺ and other transition elements. The phenomenon represents a new type of inhibition for cytochrome c oxidase. In the present paper, the conditions under which the effect is produced and reversed are described, and hypotheses to explain it are presented. A previous report from this laboratory that the effect is due to copper depletion of cytochrome c oxidase (3, 4) has been shown to be incorrect for reasons which are presented here and in an accompanying paper (5).

EXPERIMENTAL PROCEDURES AND RESULTS

Materials—Type V bovine heart cytochrome c was purchased from the Sigma Chemical Company (e.g. Lots 27B-7000, 106B-7622). Reduced cytochrome c was prepared by the method of Hemmerich and Sigwart (7); we are indebted to Dr. Andreas Zuberbuhler for the preparation. Quartz-distilled deionized water (specific conductivity, 0.9 × 10⁻⁶ mho) was used for all analytical and preparative procedures.

Ammonium sulfate (special enzyme grade, Mann Research Laboratories) was recrystallized from pure water; saturated solutions were prepared at 20°C and adjusted to pH 7.4 with ammonium hydroxide. Chemicals were obtained as follows: dibasic sodium phosphate, A.C.S. reagent grade (Matheson, Coleman and Bell); monosodium phosphate, reagent grade (Merk and Company); Tween 80 (polyoxyethylene (20) sorbitan monol-}

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model 14 recording spectrophotometer at room temperatures, with a scattered transmission accessory fitted with an RCA 6217 and window photomultiplier tube sensitive in the region from 400 to 800 nm, and a Sylvania type DXL halogen quartz high intensity light source. Quantitative analytical absorption values were measured with a Zeiss PMQ II spectrophotometer. Electron spin resonance spectra were determined with a Varian V-4500 spectrometer equipped with 100-kHz field modulation and Fieldial magnetic field control. Measurements were made near liquid nitrogen temperature, specific conditions being given in the legend to the figure. Double integration according to the method of Wyard (15) was utilized for quantitative estimation of ESR-detectable cupric copper, with CuSO₄-EDTA solutions as spin concentration standards. Total ESR-detectable copper was determined by the same method after denaturation of the enzyme solutions in the presence of p-chloromercuriphenyl-sulfonic acid and urea, according to the procedure of Beinert and Palmer (6, 16). Spectra are depicted as the first derivative of absorption of microwave power with respect to magnetic field strength. The power of incident microwave radiation at the cavity was controlled by a microwave circuit permitting continuous attenuation.

Assay of Cytochrome c Oxidase—Cytochrome c oxidase activity was measured by the polarographic method of Smith and Camerino (17), at a high, but fixed, rather than infinite, cytochrome c concentration. The procedure as described was well suited to demonstrate the bathocuproine effect. The polarographic assay mixture consisted of 0.05 M sodium phosphate buffer, pH 7.0; 50 mM sodium ascorbate, pH 7.0; 0.1 mM EDTA; 50 μM cytochrome c; and cytochrome c oxidase in appropriate dilutions in 0.1 M phosphate-1% Tween 80 buffer, pH 7.4, in a total volume of 3.0 ml. The activity measured with this system is expressed as turnover number, TN, in units of reciprocal seconds, at various oxidase concentrations.

\[
TN = \frac{\mu M \text{ O}_2 \text{ consumed per sec} \times 4}{\mu M \text{ cytochrome } a_3}
\]

The turnover number of original, reactivated, and copper-repleted oxidases were delicately dependent upon oxidase concentration in the assay medium. Maximal turnover numbers were observed at high enzyme dilutions (5 to 10 μM cytochrome a₃), but upon further dilution they decreased (Fig. 1). The final concentrations of Tween 80 in the assay mixture were about 0.06 to 0.006%. This was not critical, but the use of Tween 80 in the medium used for diluting the enzyme was. The assay was carried out immediately after diluting the stock enzyme solution with 0.1 M phosphate-1% Tween 80, pH 7.4, and immediately upon making up the reaction system in the assay cuvette, because activity diminished more than 20% in 1 hour. Under the assay conditions described here, our original enzyme preparations routinely gave turnover numbers among the highest reported for cytochrome c oxidase preparations, e.g. 400 to 750 sec⁻¹. Much lower values were observed when phosphate buffer alone or phosphate-Emasol 1130 mixtures were used for the assay medium. At the high enzyme dilutions at which maximal turnover numbers were observed, additives to the assay mixture such as phospholipid sols (cf. Reference 18), Emasols 4130, or 1130 (cf. References 19, 20) or 1 mM EDTA (cf. Reference 21) yielded lower turnover numbers, but an increase of Tween 80 in the diluent buffer from 1 to 10% had no perceptible effect. It is interesting that 5 mM EDTA is reported to inhibit the reductive titration of some cytochrome c oxidase copper (22). Bathocuproine (0.5 × 10⁻⁴ M) had no effect on the relationship between concentration of cytochrome c oxidase and its turnover number. This observation confirms the results of Griffiths and Wharton (1) and Yonetani (2) and extends them to the full range of the concentration-activity relationship under study here.

Enzyme Preparation—Our preparation was based upon the method of Fowler, Richardson, and Hatofi (13), modified for Keilin-Hartree particles instead of bovine heart mitochondria (23). The final fractionation was carried out with saturated ammonium sulfate, 0.10 ml per ml of preparation, and then 0.13 ml per ml, giving an enzyme preparation with a somewhat lower heme a to protein ratio than originally reported. This preparation was well suited to demonstrate the Cu¹-reversible bathocuproine effect. It was dissolved in Tris-sucrose-histidine buffer and could be stored for about a month without loss of activity.

The cytochrome c oxidase preparations obtained by the foregoing procedure had the following characteristics: copper to heme a ratios varied from 1.02 to 1.12; heme a to cytochrome a₃ ratios varied from 2.02 to 2.8; cytochromes b + c₁ concentrations ranged from nil to 22% of the total heme (heme a + cytochromes b and c₁) (24). Turnover numbers with respect to cytochrome a₃, measured as described, varied from 400 sec⁻¹ to 750 sec⁻¹. There was no detectable relationship between the contents of cytochrome b and c₁, and turnover number, copper content, or extent of the bathocuproine effect.

Preparation of Bathocuproine-treated Cytochrome c Oxidase—All glassware and reagents were made as copper-free as possible

\(^1\) The abbreviation used is: ESR, electron spin resonance.

\(^2\) A modification, by Dr. P. M. Nair, of a procedure already published (3).
Fig. 2. Turnover number-concentration curves of original, bathocuproine-treated, and reactivated cytochrome c oxidases. The enzymes had the following properties: A, original enzyme (No. 102), heme a to cytochrome aa ratio = 2.24; copper to heme a = 1.1; turnover number, 630 sec⁻¹; bathocuproine-treated 102a, heme a to cytochrome aa = 2.1; turnover number = 106 sec⁻¹; bathocuproine-treated 102b, heme a to cytochrome aa = 2.6; turnover number = 124 sec⁻¹; reactivated 102a, heme a to cytochrome aa = 3.1; turnover number = 532 sec⁻¹; reactivated 102b, heme a to cytochrome aa = 3.01; turnover number = 565 sec⁻¹; B, original enzyme (No. 103), heme a to cytochrome aa = 2.4; copper to heme a = 1.1; turnover number = 527 sec⁻¹; bathocuproine-treated 103, heme a to cytochrome aa = 2.4; turnover number = 96 sec⁻¹; reactivated 103, heme a to cytochrome aa = 3.01; turnover number = 866 sec⁻¹.

### Table I

**Conditions for production of Cu²⁻ reversible bathocuproine effect**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Heme a to cytochrome aa ratio</th>
<th>TN *</th>
<th>Cu²⁻ reversibility a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original enzyme (control)</td>
<td>2.36</td>
<td>666 (7 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>2.31</td>
<td>691 (7 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td>Procedure, but without bathocuproine, cytochrome c, and ascorbate</td>
<td>2.16</td>
<td>596 (6 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>585 (6 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td>Procedure, but without bathocuproine and cytochrome c</td>
<td>2.16</td>
<td>666 (5 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>2.10</td>
<td>592 (6 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td>Procedure, but without bathocuproine</td>
<td>2.27</td>
<td>263 (8 mM)</td>
<td>Not reversible</td>
</tr>
<tr>
<td></td>
<td>2.31</td>
<td>246 (14 mM)</td>
<td>Not reversible</td>
</tr>
<tr>
<td>Procedure, but without cytochrome c</td>
<td>2.20</td>
<td>124 (31.5 mM)</td>
<td>Not reversible</td>
</tr>
<tr>
<td></td>
<td>2.32</td>
<td>186 (58 mM)</td>
<td>Not reversible</td>
</tr>
<tr>
<td>Procedure as described</td>
<td>2.50</td>
<td>108 (51 mM)</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>112 (82 mM)</td>
<td>Reversible</td>
</tr>
</tbody>
</table>

* TN (sec⁻¹) the maximum turnover number, observable at the stated concentration of cytochrome c oxidase expressed as cytochrome a₃.

* Cu²⁻ reversibility, reversibility of the bathocuproine effect when the enzyme prepared as described is treated with Cu²⁺ acetonitrile, as described under “Experimental Procedures and Results.”

* Procedure: the procedure described in the text for producing bathocuproine-treated cytochrome c oxidase.
bathocuproine complex bound to the lipoprotein, the precipitate from the first ammonium sulfate fraction was separated by centrifugation, redissolved in 10 ml of cold phosphate-Tween buffer, and reprecipitated with 3 ml of neutral saturated ammonium sulfate. The reddish yellow supernatant solution was discarded, and the procedure was repeated until the supernatant solution was colorless. The final precipitate was dissolved in 0.5 ml of phosphate-Tween buffer. It contained 70 to 100% of the original heme a and was very soluble. This enzyme was readily reactivated with Cu" acetonitrile. It was stable when stored for about 1 week at -20°C. The effect of bathocuproine treatment on the turnover number of the enzyme is depicted in Fig. 2. Two examples are given to illustrate variability. Although activities in the range of 30 to 150 sec⁻¹ were observed at high enzyme concentrations (50 to 150 μm cytochrome a₃), there was a striking failure to yield the high turnover numbers characteristic of original (and CuI-reactivated) cytochrome c oxidase in the low enzyme concentration range of the assay (e.g., 5 to 10 μm cytochrome a₃); a drop to very low values was seen instead. The heme a to cytochrome a₃ and Cu to heme a ratios in these preparations were essentially unchanged from their original values. However, the preparations contained bathocuproine to heme a values of about 3.8 to 4.1 (Preparations 121 and 128). These were determined by a modification of the method of Tsudzuki, Orii, and Okunuki (26), adding Cu⁴⁺ until, upon dithionite reduction, no further increase in absorbance at 480 nm was obtained. The CuI bathocuproine concentration was determined from the absorbance at 480 nm in the difference spectrum, reduced minus oxidized, with the absorbance coefficient 12 nm⁻¹ cm⁻¹.

A number of variations upon the procedure for producing bathocuproine-inhibited cytochrome c oxidase are described in Table I. By following the procedure as described, in the absence, however, of (a) bathocuproine, cytochrome c, and ascorbate, or (b) bathocuproine and cytochrome c, only small losses in activity were observed. If the procedure was followed in the absence of (c) bathocuproine only or (d) cytochrome c only, major losses in activity were observed, and these could not be reversed by the addition of Cu⁴⁺ acetonitrile as described in the following text. Only the complete procedure as described gave rise to major losses in activity which could be reversed by Cu⁴⁺ acetonitrile. Hence, cytochrome c, bathocuproine, and ascorbate were all required to give rise to the characteristic change in the activity-concentration relationship which was Cu⁴⁺-reversible.

**Table II**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Heme a to cytochrome a₃ ratio</th>
<th>Turnover numbera</th>
<th>Optimum concentration²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original enzymes</td>
<td>2.43 ± 0.22 (11)</td>
<td>599 ± 73 (11)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>Bathocuproine-treated</td>
<td>2.35 ± 0.22 (13)</td>
<td>58 ± 11 (13)</td>
<td>133 ± 31 (13)</td>
</tr>
<tr>
<td>CuI-reactivated</td>
<td>3.32 ± 0.32 (8)</td>
<td>596 ± 74 (8)</td>
<td>17 (8)</td>
</tr>
<tr>
<td>Cu⁺⁺-reactivated</td>
<td>2.92 ± 0.53 (7)</td>
<td>577 ± 74 (7)</td>
<td>5 (7)</td>
</tr>
<tr>
<td>Copper-treated original</td>
<td>3.95 ± 0.04 (4)</td>
<td>1388 ± 213 (4)</td>
<td>2 (4)</td>
</tr>
</tbody>
</table>

*Turnover numbers were measured at a range of enzyme concentrations under the conditions described in the text. The values given refer to the highest observed values.

*The enzyme concentration (as μm cytochrome a₃) at which the highest turnover numbers were observed for each preparation. Detailed study of the effect of concentration changes around the optimal concentration was not carried out; the value for each enzyme assay was obtained from duplicate determinations of concentration-activity curves in which the initial, high, concentration was successively halved, e.g. 100, 50, 25, 19.5, 6.3, and 3.2 μm cytochrome a₃ (cf. Fig. 4, A and B).
FIG. 4. The effect of copper treatment on the turnover number-concentration curve of original cytochrome c oxidase. The results of three experiments with cytochrome c oxidase preparations 104 and 105 produced increases in turnover numbers from 480 sec⁻¹, 480 sec⁻¹, and 694 sec⁻¹ to 1168 sec⁻¹, 1267 sec⁻¹, and 1650 sec⁻¹, respectively. This figure shows a result with Preparation 105, which had a heme a to cytochrome a₃ ratio = 2.6; copper to heme a = 1.0; and turnover number = 694 sec⁻¹ (4.7 mmol cytochrome a₃). The bathocuproine (BCS)-treated oxidase from this enzyme had heme a to cytochrome a₃ = 2.2, and turnover number = 128 sec⁻¹ (67 mmol cytochrome a₃). The reactivated oxidase had heme a to cytochrome a₃ = 3.68; turnover number = 756 sec⁻¹ (4.0 mmol cytochrome a₃). The copper-repleted oxidase had heme a to cytochrome a₃ = 4.3; turnover number = 1650 sec⁻¹ (1.9 mmol cytochrome a₃). The assays were also carried out in the presence of 0.5 × 10⁻⁴ M bathocuproine disulfonate and in the presence of catalase (3 mg/3 ml assay mixture). Curve A, original enzyme; Curve B, bathocuproine-treated oxidase preparation 105a; Curve C, bathocuproine-treated oxidase preparation 105b; Curve D, Cu²⁺-reactivated preparation 105a, Curve E, copper-repleted original oxidase.

Reactivation of Bathocuproine-treated Cytochrome c Oxidase—Bathocuproine-treated enzyme was diluted to 10 μM with respect to heme a in 0.1 M phosphate-1% Tween 80 buffer, pH 7.4, and to this solution (usually 10 ml) was added Cu²⁺ acetonitrile (0.001 M in acetonitrile) in a ratio of 3 g atom per mole of heme a. The mixture was incubated at 4° for 90 min; the enzyme was precipitated with neutral saturated ammonium sulfate (0.3 ml per ml); and the precipitate was recovered by centrifugation, redissolved in phosphate-Tween 80 buffer, and reprecipitated with neutral ammonium sulfate. The enzyme was finally dissolved in phosphate Tween buffer. It was stable in this buffer for 3 to 4 hours at 0°; the use of other buffers for storage of reconstituted enzyme was not studied. Most of the starting heme a (80 to 90%) was recovered in this process.

Turnover number-concentration curves for reactivated cytochrome c oxidase are shown in Fig. 2. The activity-dilution curve was very similar to that of original enzyme. When assays were carried out in the presence of catalase (3 mg/3 ml of assay mixture) or 0.5 × 10⁻⁴ M bathocuproine disulfonate, no effect upon the turnover number-concentration relationship was observed. Thus, it is improbable that copper, nonenzymically combined, acted as a catalyst of cytochrome c autoxidation (cf. Reference 26) or that hydrogen peroxide was a primary product of reaction.

The effect of Cu²⁺ acetonitrile concentration on the reactivation reaction was studied systematically by varying the Cu²⁺ to heme a ratio in the system. The results are shown in Fig. 3. Optimal reactivation was obtained with a ratio of 3. The effect of time on the reactivation reaction was also examined; optimal results were obtained at 90 min, the activity of the product subsequently falling off.

Reactivation of bathocuproine-treated oxidase also occurred when the enzyme was treated, under reactivation conditions, with cobaltous ions. The phenomenon is described in Table II. The activity of the cobalt-reactivated enzyme was unaffected by the presence of catalase (1 mg per ml, in the final assay mixture) or 0.5 × 10⁻⁴ M bathocuproine, making autoxidation an unlikely basis of the observed reactivation of O₂ consumption.

Reactivation of Bathocuproine-treated Cytochrome c Oxidase—The properties of a representative series of bathocuproine-treated and Cu²⁺-reactivated cytochrome c oxidases are shown in Fig. 2. The activity-dilution curve was very similar to that of original enzyme. When assays were carried out in the presence of catalase (3 mg/3 ml of assay mixture) or 0.5 × 10⁻⁴ M bathocuproine, no effect upon the turnover number-concentration relationship was observed. Thus, it is improbable that copper, nonenzymically combined, acted as a catalyst of cytochrome c autoxidation (cf. Reference 26) or that hydrogen peroxide was a primary product of reaction.

The effect of Cu²⁺ acetonitrile concentration on the reactivation reaction was studied systematically by varying the Cu²⁺ to heme a ratio in the system. The results are shown in Fig. 3. Optimal reactivation was obtained with a ratio of 3. The effect of time on the reactivation reaction was also examined; optimal results were obtained at 90 min, the activity of the product subsequently falling off.

Optical Spectra of Original, Bathocuproine-treated, and Cu²⁺-reactivated Cytochrome c Oxidase—Absolute spectra of original, bathocuproine-treated, and Cu²⁺-reactivated cytochrome c oxidasess in their oxidized, reduced, and CO-complex forms were essentially the same as reported (3). All forms of the reactivated oxidase showed absorption in the 480 μm region, corresponding to absorption of Cu²⁺ bathocuproine disulfonate from bathocuproine adsorbed to the enzyme and not removed by subsequent dialysis. The magnitude of the 480 μm absorption corresponded to between 1.2 and 1.8 moles of Cu²⁺-bathocuproine per mole of heme a (Preparations 121, 124, 127, and 128).

We reported that the near infrared absorption band of cytochrome c oxidase was diminished upon bathocuproine treatment (4). We have now found that when the Zeiss PMQ II spectrophotometer is used for these measurements, the results are variable, whereas, when the Cary model 14 with scattered transmission accessory is used, the results are consistent and show no diminution of the 830 μm band. This suggests that earlier results were due to an apparent loss of 830 μm band absorption because of light scattering.
Enzyme Preparation—Our enzyme preparations had a lower heme a to protein ratio than that obtained by Hafei and his co-workers (13) (3.3 to 6.5 mmoles as opposed to 5.4 to 8.7 mmoles of heme a per mg of protein), variable but higher contents of cytochromes b and a₃, and very high turnover numbers under the conditions of assay which we describe. The turnover numbers were comparable to those shown by Keilin-Hartree particles (cf. Reference 23), but the ratios of heme a to protein were about 2.5 to 5 times higher than ratios in the particles (23, 27).

Enzyme Assay—The primary effect of Bathocuproine treatment and Cu⁺ reactivation of cytochrome c oxidase was decrease and increase, respectively, of turnover numbers at high dilutions in the presence of Tween 80 (Table II). The essential aspect of the assay procedure was initial exposure of oxidase to 1% Tween 80, followed by dilution in assay medium containing no other surface-active agent, and the use of low concentrations of enzyme (5 to 10 μM) in the final assay mixture. Under these conditions, high turnover numbers were regularly observed with original and reactivated oxidases (even higher with copper-repleted oxidase). These turnover numbers approached or exceeded values observed by other investigators with Keilin-Hartree heart muscle particles and intact mitochondria (17, 28–30). In addition, these turnover numbers probably approximate values computed for infinite cytochrome c concentrations, because at very high dilutions of cytochrome c oxidase, turnover numbers measured under conditions similar to those used here are the same as values computed for infinite cytochrome c concentration (21).

Cytochrome c oxidase occurs as a membrane-bound enzyme which gives rise to solubilized preparations of varying polydispersity. The measurement of its activity in its native and “purified” states has accordingly presented difficult problems which were apparently solved by the use of surface-active agents in the assay system, e.g. Emasol 1130 (31, 32), Emasol 4130 (21), bovine serum albumin (33), phospholipid (18, 34), sodium deoxycholate (28), Tween 80 (3), and others. Of these, Tween 80, as used in the present study, appears to give the highest turnover numbers yet observed for solubilized cytochrome c oxidase preparations. For example, a preparation assayed at the same temperature and pH, but in the presence of Emasol 1130 and 1 mM EDTA (cf. Reference 21) instead of initial exposure to 1% Tween 80 followed by dilution, gave maximum turnover numbers of 200 sec⁻¹ (40 μM cytochrome a₃) to be compared with 650 sec⁻¹ (5 μM cytochrome a₃) by the method described here. In fact, Emasol 1130 and 1 mM EDTA diminish cytochrome c oxidase activity when measured in our assay system. It is interesting that Smith and Newton (28) have observed turnover numbers nearly as high as those we report, when concentrated preparations of heart muscle particles were treated with sodium deoxycholate, then diluted for assay. The principle involved in their assay system and ours appears to be the same.

The mechanism by which surface-active agents activate preparations of mammalian cytochrome c oxidase has been the subject of extensive study and discussion (17–21, 25, 28, 29, 32, 37, 38). It is generally accepted that a primary effect of surface-active agents upon both particulate (28) and “purified” states (39) is to make the enzyme more accessible to reduced cytochrome c, presumably by altering conformation or state of aggregation (18, 32, 37, 40, 41). Direct evidence for the capacity of mammalian cytochrome c oxidase to exist in several states of aggregation has been obtained by Criddle and Bock (42), Take-mori and co-workers (43), and Orii and Okumuki (37).

We suggest that the concentration-turnover relationship for our enzyme in the presence of Tween 80 arises in part from a disaggregation of enzyme between concentrations of 100 μM and 5 μM cytochrome a₃. Under these conditions, apparently an accumulation of an unstable unit aggregate of maximal activity occurs in the low oxidase concentration region of assay. Based upon the estimations of molecular weight by Orii and Okumuki (37), the active unit may be an aggregate consisting of a cytochrome a molecule, a cytochrome a₃ molecule, and two copper proteins (cf. Reference 44). It is of interest that the dilute oxidase gives rise to high turnover numbers which are also characteristic of intact mitochondria (30). The structural basis of this resemblance remains to be determined.

Changes in specific activity of polymeric enzymes upon dilution, due to changes in state of aggregation, are known in other cases. For example, Waksman and Roberts (45) found that γ-aminobutyric α-ketoglutaric acid transaminase (mouse brain) increased in activity upon dilution in a manner similar to that reported here for cytochrome c oxidase.

The essential characteristic of the Bathocuproine effect is the failure of the treated oxidase to show significant activity at dilutions in the 5 to 10 μM range, whereas appreciable activities are shown at higher concentrations. It seems reasonable to ascribe this property of Bathocuproine-treated oxidase to an incapacity to disaggregate into unit aggregates of maximal activity because the effect is observed in the concentration-activity relationship. The questions then arise: why is the concentration-activity relationship changed in this manner, and why is the change reversed by Cu⁺ or Co²⁺? Several hypotheses present themselves, which relate to the chemistry of the equilibrium among the subunits of the oxidase and to the nature of the rate-limiting steps (cf. Reference 23). The essential points are that Bathocuproine-treated oxidase is more accessible to reduced cytochrome c, and that the enzyme treated with Bathocuproine is capable of showing the dispersion phenomenon at 5 to 10 μM cytochrome a₃ concentration.
Cytochrome Oxidase: Bathocuproine Effect

Turnover numbers of cytochrome c oxidase preparations calculated with respect to cytochrome a₃, heme a, and cytochrome a

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Turnover number$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytochrome a₃</td>
</tr>
<tr>
<td>Original enzyme</td>
<td>599 ± 73, 5 (11)</td>
</tr>
<tr>
<td>Bathocuproine-treated oxidase$^a$</td>
<td>133 ± 21, 58 (18)</td>
</tr>
<tr>
<td>Cu$^2+$-reactivated enzyme</td>
<td>596 ± 74, 5 (8)</td>
</tr>
<tr>
<td>Cu$^{2+}$ reactivated enzyme</td>
<td>577 ± 74, 5 (7)</td>
</tr>
<tr>
<td>Copper-repleted oxidase</td>
<td>1338 ± 213, 2 (4)</td>
</tr>
</tbody>
</table>

$^a$ µM O₂ consumed per second, × 4/µM concentration of reference substance.

* Concentration of cytochrome a was calculated from the difference between heme a and cytochrome a₃ concentrations.

* Turnover numbers of bathocuproine-treated oxidase were calculated for optimal concentrations of enzyme, which were about 10-fold higher than the optimal concentrations for the other enzyme preparations. In the region of 5 mM cytochrome a₃ concentrations, bathocuproine-treated oxidase showed little or no enzymic activity.

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### Acknowledgments

We wish to thank Dr. P. M. Nair for the modified method of bathocuproine treatment, and some of the ESR measurements, which are reported in this study. Dr. David Wharton provided friendly and very helpful cooperation in phases of the research dealing with reproducibility of the bathocuproine effect, and its relationship to copper. Mrs. Annette Johnson and Mrs. Nancy Avedovech gave us essential support for the study with their careful preparative work, and we acknowledge their help with gratitude.

### REFERENCES

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