Cytochrome c Oxidase

THE BATHOCUPROINE EFFECT*

H. S. Mason and K. Ganapathy

From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201

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 Orie and Morrison (6). Cuprous acetonitrile perchlorate 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° and adjusted to pH 7.4 with ammonium hydroxide. Chemicals were obtained as follows: dibasic sodium phosphate, A.C.S. reagent grade (Merk and Company); Tween 80 (polyoxyethylene (20) sorbitan monol-}

Received for publication, March 28, 1969

* This study was supported by Grants AM-07180, from the United States Public Health Service, and E-225, from the American Cancer Society, for which we are indebted.
model 14 recording spectrophotometer at room temperatures, with a scattered transmission accessory fitted with an RCA 6217 end 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-chloromercuriphenylsulfonic acid and urea, according to the procedure of Beinert and Palmer (5, 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 pM 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.

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

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 mM cytochrome c); 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 sds (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 \times 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, Richardsson, and Hatefi (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

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1 The abbreviation used is: ESR, electron spin resonance.

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Fig. 1. The relationship between oxidase concentration and turnover number of cytochrome c oxidase preparations assayed by the procedure described in the text. The figure depicts the turnover number-concentration relationship for three cytochrome c oxidase preparations having copper to heme a ratios of 1.1 (A); 1.1 (B); and 1.1 (C).
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 a$_3$ ratio = 2.24; copper to heme a = 1.1; turnover number, 630 sec$^{-1}$; bathocuproine-treated 102a, heme a to cytochrome a$_3$ = 2.1; turnover number = 106 sec$^{-1}$; bathocuproine-treated 102b, heme a to cytochrome a$_3$ = 2.6; turnover number = 124 sec$^{-1}$; reactivated 102a, heme a to cytochrome a$_3$ = 3.1; turnover number = 527 sec$^{-1}$; bathocuproine-treated 103, heme a to cytochrome a$_3$ = 2.4; turnover number = 96 sec$^{-1}$; reactivated 103, heme a to cytochrome a$_3$ = 3.01; turnover number = 866 sec$^{-1}$.

TABLE I

Conditions for production of Cu$^2+$-reversible bathocuproine effect

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Heme a to cytochrome a$_3$ ratio</th>
<th>TN$^{a}$</th>
<th>Cu$^2+$ reversibility$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original enzyme (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procedure, but without bathocuproine, cytochrome c, and ascorbate</td>
<td>2.36</td>
<td>666 (7 mUM)</td>
<td>Reversible</td>
</tr>
<tr>
<td>Procedure, but without bathocuproine and cytochrome c</td>
<td>2.16</td>
<td>596 (6 mUM)</td>
<td>Not reversible</td>
</tr>
<tr>
<td>Procedure as described</td>
<td>2.27</td>
<td>263 (8 mUM)</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 a$_3$.

* Cu$^2+$ reversibility, reversibility of the bathocuproine effect when the enzyme prepared as described is treated with Cu$^+$ 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\(^{2+}\) acetonitrile. It was stable when stored for about 1 week at -20\(^\circ\)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 mM cytochrome \(a_3\)), there was a striking failure to yield the high turnover numbers characteristic of original (and Cu\(^{2+}\)-reactivated) cytochrome \(c\) oxidase in the low enzyme concentration range of the assay (e.g. 5 to 10 mM cytochrome \(a_3\)); a drop to very low values was seen instead. The heme \(a\) to cytochrome \(a_3\) 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 (25), adding Cu\(^{2+}\) until, upon dithionite reduction, no further increase in absorbance at 480 nm was obtained. The Cu\(^{2+}\) bathocuproine concentration was determined from the absorbance at 480 nm in the difference spectrum, reduced minus oxidized, with the absorbance coefficient 12 mM\(^{-1}\) 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 Cu\(^{2+}\) 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 Cu\(^{2+}\) acetonitrile. Hence, cytochrome \(c\), bathocuproine, and ascorbate were all required to give rise to the characteristic change in the activity-concentration relationship, which was Cu\(^{2+}\)-reversible.

![Fig. 3. Effect of Cu\(^{2+}\) acetonitrile concentration on the reactivation of cytochrome \(c\) oxidase. Turnover numbers are shown as a function of concentration of oxidase reactivated as described in the text, but with varying Cu\(^{2+}\) to heme \(a\) ratios. The original enzyme (Curve A) had the following characteristics: heme \(a\) to cytochrome \(a_3\) ratio = 2.38; copper to heme \(a\) = 1.1. The bathocuproine-treated enzyme (Curve B) had the following characteristics: heme \(a\) to cytochrome \(a_3\) = 2.5. The reactivated enzymes had the following characteristics: heme \(a\) to cytochrome \(a_3\) = 2.5; Curve D, Cu\(^{2+}\) to heme \(a\) during reactivation = 0.1; product, heme \(a\) to cytochrome \(a_3\) = 2.5; Curve E, Cu\(^{2+}\) to heme \(a\) during reactivation = 1.0; product, heme \(a\) to cytochrome \(a_3\) = 2.78; Curve F, Cu\(^{2+}\) to heme \(a\) during reactivation = 3.0; product, heme \(a\) to cytochrome \(a_3\) = 3.27.](http://www.jbc.org/)

### Table II

**Characteristics of original, bathocuproine-treated, reactivated, and copper-treated cytochrome \(c\) oxidases**

The numbers in parentheses refer to the mean value, one standard deviation, and the number of experiments. The analytical methods are described in the text.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Heme (a) to cytochrome (a_3) ratio</th>
<th>Turnover number(^a)</th>
<th>Optimum concentration(^b)</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>Cu(^{2+})-reactivated</td>
<td>3.32 ± 0.32 (8)</td>
<td>596 ± 74 (8)</td>
<td>17 (8)</td>
</tr>
<tr>
<td>Cu(^{2+})-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>

\(^a\) 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.

\(^b\) The enzyme concentration (as mM cytochrome \(a_3\)) 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 mM cytochrome \(a_3\) (cf. Fig. 4, A and B).
in phosphate Tween buffer. It was stable in this buffer for 3 to 4 hours at 0°C; 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 \( \times \) \( 10^{-4} \) 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 CuI acetonitrile concentration on the reactivation reaction was studied systematically by varying the CuI 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 \( \times \) \( 10^{-4} \) M bathocuproine, making autoxidation an unlikely basis of the observed reactivation of O2 consumption.

Copper Treatment of Original Enzyme—We have reported that original enzyme treated with CuI acetonitrile under reactivation conditions showed remarkable increases in turnover number (3). These experiments have now been repeated, and the results have been depicted as turnover number-concentration curves (Fig. 4).

Copper treatment of original enzyme consistently yielded products with turnover numbers in the range from 1000 sec\(^{-1}\) to 1600 sec\(^{-1}\). These were unaffected by the presence of catalase or bathocuproine disulfonate in the assay medium.

Optical Spectra of Original, Bathocuproine-treated, and CuI-reactivated Cytochrome c Oxidase—Absolute spectra of original, bathocuproine-treated, and CuI-reactivated cytochrome c oxidases in their oxidized, reduced, and CO-complex forms were obtained at 90 min, the activity of the product subsequently falling off.

The effect of CuI acetonitrile concentration 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 \( \times \) \( 10^{-4} \) M bathocuproine, making autoxidation an unlikely basis of the observed reactivation of O2 consumption.

Optical Spectra of Original, Bathocuproine-treated, and CuI-reactivated Cytochrome c Oxidase—Absolute spectra of original, bathocuproine-treated, and CuI-reactivated cytochrome c oxidases 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 nm region, corresponding to absorption of CuI bathocuproine disulfonate arising from the reaction of CuI acetonitrile with bathocuproine adsorbed to the enzyme and not removed by subsequent dialysis. The magnitude of the 480 nm absorption corresponded to between 1.2 and 1.8 moles of CuI-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 nm band. This suggests that earlier results were due to an apparent loss of 830 nm band absorption because of light scattering.

Composition of Bathocuproine-treated and CuI-reactivated Cytochrome c Oxidase—The properties of a representative series of bathocuproine-treated and CuI-reactivated cytochrome c ox-
Electron Spin Resonance Spectra of Original, Bathocuproine-treated, and Reactivated Cytochrome c Oxidase—The ESR spectra of original, bathocuproine-treated, and Cu$^+$-reactivated cytochrome c oxidase in the g = 2 region were essentially of the same shape and position at the same microwave powers, at $-190^\circ$. All were of the ESR-detectable native copper type. The ratio of ESR-detectable copper to heme a did not change as a result of bathocuproine treatment. Thus, a prior result (3) could not be repeated. There were no essential differences in the satura-
bility of the g = 2 signals up to 150 milliwatts among the three forms of the enzyme.

**DISCUSSION**

**Enzyme Preparation**—Our enzyme preparations had a lower heme a to protein ratio than that obtained by Hatfield and his co-workers (13) (5.5 to 6 mmol as opposed to 5.4 to 8.7 mmol of heme a per mg of protein), variable but higher contents of cytochromes b and c$_1$, 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 mmol cytochrome a$_2$ 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 deoxy-
cholate (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 mmol cytochrome a$_2$) to be compared with 650 sec$^{-1}$ (5 mmol cytochrome a$_2$) 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 cyto-
chome c, presumably by altering conformation or state of aggre-
gation (15, 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 Cridde 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 mmol and 5 mmol cytochrome a$_2$. 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 a$_3$ 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 dilu-
tion, 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 mmol 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 concentra-
tion-activity relationship changed in this manner, and why is the change reversed by Cu$^+$ or Co$^{12}$? Several hypotheses pre-
sent themselves, which relate to the chemistry of the equilibrium among the subunits of the oxidase and to the nature of the rate-
was more recently emphasized by Gibson and Wharton (36). In the experimental work on which their conclusion was based, the minimum concentration of cytochrome c oxidase was 700 mmol heme a$_2$ about 50-fold greater than the concentration range in which we observe the activation phenomenon. In addition, it is not certain that the enzyme used by Gibson and Greenwood was capable of showing the dispersion phenomenon at 5 to 10 mmol cytochrome a$_2$ concentration.
Turnover numbers of cytochrome c oxidase preparations calculated with respect to cytochrome a3, heme a, and cytochrome a

The columns under each heading refer to the mean value of the turnover number, one standard deviation of the turnover numbers measured, the mean concentration (millimicromolar) at which the turnover numbers were measured, and the number of preparations upon which the means and the standard deviations were based.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Turnover number*</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</td>
<td>133 ± 21, 58 (18)</td>
</tr>
<tr>
<td>CuI-reactivated enzyme</td>
<td>596 ± 74, 5 (8)</td>
</tr>
<tr>
<td>CoII-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>

* μM O2 consumed per second, x 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.

**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.

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