A SPECTROPHOTOMETRIC STUDY OF THE COMPETITION OF 
METHEMOGLOBIN AND CYTOCHROME OXIDASE FOR 
CYANIDE IN VITRO 

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Although the efficacy of induced methemoglobinemia in cyanide poisoning 
has been demonstrated both therapeutically and prophylactically (1),
the tissue reactions involved in the protection of animals from the lethal effects 
of the cyanide ion by methemoglobin are only imperfectly understood. 
Presumably, cyanide interferes with cellular respiration by forming a 
slightly dissociable complex with cytochrome oxidase (2–4). Kobert (5) 
first showed that methemoglobin combines with cyanide to form cyanmethemoglobin. The often reported antidotal action of methemoglobin-forming 
substances in cyanide poisoning may then be regarded as a competitive 
reaction between methemoglobin and cytochrome oxidase for cyanide ion. 
It is the purpose of the present work to determine whether this competition 
may be demonstrated \textit{in vitro} and to obtain some idea of the comparative 
dissociabilities of cyanmethemoglobin and cytochrome oxidase-cyanide 
complex. 

EXPERIMENTAL 

A spectrophotometric technique based upon the rate of oxidation of re-
duced cytochrome $c$ was used for the measurement of cytochrome oxidase 
activity (6, 7). Since reduced cytochrome $c$ has a sharp absorption peak at 
5500 Å, the rate of oxidation can be measured by determining the change in 
absorption of monochromatic light at that wave-length. 

Ideally, the competition of cytochrome oxidase and methemoglobin 
should be studied in a system in which the various components are present 
in known concentrations. In the present work, practically pure crystalline 
methemoglobin and a purified preparation of cytochrome $c$ were used. No 
purified preparation of cytochrome oxidase was available. A very dilute 
homogenate of brain was used as the source of cytochrome oxidase. 

Adult male rats, weighing about 300 gm., were decapitated and the brains 
quickly removed. In each experiment, the brain was rapidly weighed on a 
torsion balance, transferred to a Waring blender, and enough iced distilled 
water added to yield a 2 per cent homogenate. The tissue was homogenized 

\footnote{Jandorf, B. J., and Bodansky, O., \textit{J. Ind. Hyg. and Toxicol.}, in press.}
for 2 minutes, after which it was strained through several layers of surgical
gauze to remove gross particles. This stock homogenate was kept refriger-
ated at all times. For any experiment, 5.0 ml. of the stock homogenate
were pipetted into a tube. Water or a solution of sodium cyanide was
added to a final volume of 10.0 ml.; 0.04 or 0.05 ml. of this diluted homoge-
nate was used in each run. The cyanide stock solution prepared from
sodium cyanide was made up in a concentration of 0.01 M and checked for
cyanide content at various intervals by silver chloride titration.

Cytochrome oxidase activity was measured in the Beckman spectropho-
tometer at 5500 Å, and a nominal slit width of 1.9 μm. At this wave-
length, reduced cytochrome has a sharp absorption maximum. As the re-
duced cytochrome is oxidized, the density decreases; the rate of decrease is
a measure of the oxidase activity. Oxidized cytochrome itself shows some
absorption at 5500 Å, so that the observed values really measure the de-
crease in density due to the disappearance of reduced cytochrome as well
as a slight increase due to the accumulation of oxidized cytochrome. If the
initial concentration of cytochrome is known, as well as the absorption
coefficients of reduced and oxidized cytochrome, it is possible from the ob-
served values to calculate the concentrations of either component at any
time. The fundamental equations have been derived by Haas, Horecker,
and Hogness (6), and by Altschul, Abrams, and Hogness (7), and were used
in the present paper to calculate the rate of disappearance of the reduced
cytochrome. The reaction velocities are expressed in terms of the mono-
molecular reaction constants.

Cytochrome c was prepared from beef heart, according to the method of
Keilin and Hartree (8); crystalline methemoglobin, used in many of the
experiments to be described, was prepared from guinea pig blood, according
to the method of Warburg and Reid (9). From one sample of blood (ap-
proximately 30 ml.), two harvests of crystals were obtained. The first
assayed 99.2 per cent methemoglobin of the total pigment, the second 95.0
per cent methemoglobin of the total pigment.

For any day's experiments, a solution containing cytochrome c and a con-
centration of phosphate buffer that would give a concentration of 0.01 M and
a pH of 7.4 in the final reaction mixture was used. The cytochrome c was
completely reduced with several crystals of dry sodium hydrosulfite, the
excess hydrosulfite oxidized by aeration, and enough water added to give a
volume of 2.95 or 2.96 ml. The density was then read at 5500 Å, after
which 0.05 or 0.04 ml., respectively, of the diluted homogenate was added;
the contents of the vessel were mixed and timed readings taken, beginning 1
minute after the addition of the enzyme and continuing at 30 second inter-
vals for from 3 to 8 minutes. Correction was made for the slight initial
increase in density due to the turbidity of the enzyme source by measuring
the turbidity of the homogenate in distilled water.
In the experiments in which the effect of methemoglobin on the effect of cyanide inhibition was studied, a methemoglobin solution was substituted for an equal volume of water. All final volumes were, therefore, maintained at 3.0 ml. Methemoglobin itself absorbs at 5500 A, so that, in those experiments in which it was used, the observed densities were corrected before the changes in cytochrome c concentration were calculated. In several experiments, preformed cyanmethemoglobin was used. This was prepared by reaction of methemoglobin and cyanide in amounts equivalent to those present in the final reaction mixture to which these substances were added separately. All cyanide concentrations indicated in the text are final concentrations in the reaction mixtures.

Since the Beckman spectrophotometer has no mechanism for keeping the reaction vessels at constant temperature, temperatures of the reaction mixture were recorded at the end of each determination. Within any series of experiments on the same day the variation was never greater than 1.5°.

**Results**

Fig. 1 shows the typical effects of cyanide ion on the rate of oxidation of cytochrome c by cytochrome oxidase, as well as the effect of methemoglobin on the activity of the oxidase partially inhibited by cyanide. The lowest curve shows that a concentration of $3.26 \times 10^{-5}$ M cyanide decreased the reaction velocity constant 85 per cent. The presence of $1.32 \times 10^{-5}$ M methemoglobin (calculated on the basis of a molecular weight of 17,000 for methemoglobin) in the cyanide-inhibited system did not affect the rates for the first 2 minutes of the reaction; thereafter, the rate increased so that the inhibition, as judged by the reaction velocity constant, was 77 per cent of the activity of the control system. This indicated that methemoglobin reversed cyanide inhibition.

Fig. 1 also shows that an amount of cyanmethemoglobin present, equivalent to $1.32 \times 10^{-5}$ M methemoglobin, had no initial effect on the activity of the oxidase. However, after 2 minutes, the rate of oxidation began to decrease; the final rate of oxidation represented a 79 per cent inhibition of the control rate.

Table I shows that $1.32 \times 10^{-5}$ M methemoglobin restored a cyanide-inhibited system from 24 to 35 per cent of the original activity, whereas $2.64 \times 10^{-5}$ M restored it to 50 per cent and $3.96 \times 10^{-5}$ M to 77 per cent of the original activity. It was not possible to study higher concentrations because of increased density of the solutions and the limitations of the instrument. Control experiments carried out simultaneously showed that the corresponding amounts of methemoglobin did not affect the rates of oxidation of cytochrome c by cytochrome oxidase in the absence of cyanide.

In the experiment described in Table I, the oxidase content and cyanide concentrations were kept constant and the methemoglobin concentration
varied. In the series of experiments shown in Fig. 2, the oxidase and methemoglobin levels were maintained constant and the cyanide concentrations were varied. Throughout the range of cyanide concentrations between approximately $5 \times 10^{-5}$ M and $1 \times 10^{-9}$ M, $1.32 \times 10^{-5}$ M methemoglobin produced the same degree of reversal of inhibition, approximately

![Graph showing the effect of methemoglobin, cyanide, and cyanmethemoglobin on brain cytochrome oxidase activity.](http://www.jbc.org/)

Fig. 1. The effect of methemoglobin, cyanide, and cyanmethemoglobin on brain cytochrome oxidase activity. The final reaction mixture had a volume of 3.0 ml. and contained 0.05 ml. of 1 per cent brain homogenate, $3.75 \times 10^{-4}$ M cytochrome, and 0.01 M phosphate buffer, pH 7.4. O shows the reaction without any additions; X, the effect of $3.26 \times 10^{-4}$ M cyanide; X, the effect of $1.32 \times 10^{-5}$ M methemoglobin in the presence of $3.26 \times 10^{-4}$ M cyanide; ● shows the effect of $1.32 \times 10^{-4}$ M preformed cyanmethemoglobin on the reaction in the absence of added cyanide. The figures in per cent represent the degree of inhibition.

10 per cent of the original activity. In other words, in the presence of this amount of methemoglobin, approximately 5 times as much cyanide was required to produce a given degree of inhibition as in the absence of methemoglobin. Thus, 50 per cent inhibition was produced by $2 \times 10^{-8}$ M cyanide while, in the presence of $1.32 \times 10^{-8}$ M methemoglobin solution, the same inhibition was produced by $8.9 \times 10^{-8}$ M cyanide.
Fig. 2. The effect of varying amounts of cyanide on the activity of brain cytochrome oxidase in the presence and absence of methemoglobin. The final reaction mixture had a volume of 3.0 ml. and contained 0.04 ml. of 1 per cent brain homogenate, 2.305 \times 10^{-6} \text{M} cytochrome, 0.01 \text{M} phosphate buffer, pH 7.4. O shows the effect of varying concentrations of cyanide; ▲, the effect of the same concentrations of cyanide in the presence of 1.32 \times 10^{-5} \text{M} methemoglobin.

**Table I**

**Effect of Varying Amounts of Methemoglobin on Cyanide Inhibition of Brain Cytochrome Oxidase Activity**

The final reaction mixture had a volume of 3.0 ml. and contained 0.05 ml. of a 1 per cent brain homogenate (100 \gamma dry weight of tissue), 0.01 \text{M} phosphate buffer pH 7.4, and 2.69 \times 10^{-6} \text{M} cytochrome.

<table>
<thead>
<tr>
<th>Composition of reaction mixture</th>
<th>Reaction velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c + brain homogenate</td>
<td>K*</td>
</tr>
<tr>
<td>&quot; &quot; + &quot; &quot; + 1.13 \times 10^{-6} \text{M cyanide}</td>
<td>0.210</td>
</tr>
<tr>
<td>&quot; &quot; + &quot; &quot; + cyanide + 1.32 \times 10^{-5} \text{M methemoglobin}</td>
<td>0.085</td>
</tr>
<tr>
<td>Cytochrome c + brain homogenate + cyanide + 2.64 \times 10^{-8} \text{M methemoglobin}</td>
<td>0.120</td>
</tr>
<tr>
<td>Cytochrome c + brain homogenate + cyanide + 3.96 \times 10^{-8} \text{M methemoglobin}</td>
<td>0.185</td>
</tr>
</tbody>
</table>

* Monomolecular reaction constant calculated as in Fig. 1 after it had attained a constant maximal value.
DISCUSSION

In none of the experiments described was more than 0.05 ml. of a 1 per cent brain homogenate used as a source of enzyme. This quantity in no case corresponded to more than 120 γ of dry tissue, of which only a small part is cytochrome oxidase. This conclusion is based on the observation that such homogenates have been shown to contain varying quantities of numerous enzymes found in animal tissues. Since no pure cytochrome oxidase preparations were available from which to make molecular weight calculations, it is very difficult to know precisely how much oxidase competes with a known amount of methemoglobin for available cyanide.

However, an estimate of the competition between methemoglobin and cytochrome oxidase for the cyanide ion may be obtained in the following way. On the assumptions that in the incompletely inhibited enzyme system practically all of the cyanide ion is combined, that the concentration of the free cyanide ion is negligible, and that cyanide combines mole for mole with the oxidase iron, the total amount of oxidase in terms of moles of iron may be estimated from the cyanide concentration. For example, at $1 \times 10^{-4}$ M cyanide, 76 per cent of the oxidase is inactivated; the order of magnitude of oxidase iron would be $100/76 \times 1 \times 10^{-6}$, or approximately $1.3 \times 10^{-5}$ M. In the experiments recorded in Table I, the methemoglobin concentrations ranged from $1.32 \times 10^{-5}$ to $3.96 \times 10^{-5}$ M methemoglobin. At a methemoglobin-cytochrome oxidase ratio of 10:1, 11 per cent of the original activity was restored. At ratios of 20:1 and 30:1, 26 to 49 per cent of the original activities were restored. These results suggest that the cytochrome oxidase-cyanide complex is less dissociable than the cyanmethemoglobin. Moreover, as has been shown in this work (Fig. 1), cyanmethemoglobin, added to a cytochrome-cytochrome oxidase system, apparently dissociates sufficiently so that the resultant cyanide ions can inhibit cytochrome oxidase activity.

In vivo, when methemoglobinemia is induced, either prophylactically or therapeutically, to counteract the effects of cyanide ion, the latter may be considered to participate in the three following reactions:

\[
\begin{align*}
CN^- + \text{cytochrome oxidase} & \rightleftharpoons \text{cytochrome oxidase cyanide} \quad (1) \\
CN^- + \text{methemoglobin} & \rightleftharpoons \text{cyanmethemoglobin} \quad (2) \\
CN^- + S \text{ compounds} & \rightleftharpoons \text{CNS}^- \quad (3)
\end{align*}
\]

When methemoglobinemia is induced prophylactically (i.e. before cyanide is administered), Reaction 2 takes place in the blood stream and the amount of cyanide ion reaching the tissues is greatly decreased. When methemoglobinemia is induced after administration of cyanide and a therapeutic result is obtained, it may be assumed that methemoglobin has combined
with enough cyanide to cause Reaction 1 to shift to the left and liberate sufficient free cytochrome oxidase to permit tissue respiration to be resumed. Reaction 3 represents a pathway for the conversion of free cyanide ion to the non-toxic thiocyanate. It has been shown that administered cyanide is excreted almost completely in the urine as thiocyanate (10).

SUMMARY

1. Experiments have been performed on the in vitro competition between methemoglobin and cytochrome oxidase in brain homogenates for cyanide ion.

2. It is shown that methemoglobin can reverse the cyanide inhibition of cytochrome oxidase activity. The extent of the reversal was studied at varying concentrations of methemoglobin and cyanide. It is shown that in a suitable in vitro system preformed cyanmethemoglobin dissociates to give sufficient free cyanide ion to combine with cytochrome oxidase.

3. The in vivo implications of the above observations are discussed briefly.

BIBLIOGRAPHY

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