THE CYTOCHROME c-AZIDE COMPLEX

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Since both cyanide and azide have been shown to block the oxidation of cytochrome c by cytochrome oxidase (1), it has been generally accepted that the toxicity of these substances is due to this property. A considerable amount of evidence has accumulated, however, indicating that azide and cyanide do not inhibit respiration in a parallel manner under all conditions. Thus Stannard (2) was able to demonstrate that only a fraction of the respiration of stimulated frog muscle is sensitive to azide and that the azide-insensitive fraction is identical with the respiration of the resting muscle. Both resting and activity respiration, on the other hand, are completely inhibited by cyanide.

Fischer, Henry, and Low (3) found similarly that the extra respiration resulting on fertilization of the sea-urchin egg is inhibited by azide, while the respiration of the unfertilized egg is not azide-sensitive. With respect to the action of cyanide on this system there is some confusion, some workers (4, 5) having reported that the respiration of the unfertilized egg is cyanide-insensitive. However, Robbie (6) attributes these findings to a failure to maintain the cyanide concentration in the manometric experiments and to a secondary oxygen uptake induced by the higher concentrations of cyanide. He presents evidence indicating that the respiration of both the unfertilized and fertilized egg is cyanide-sensitive.

To account for this fundamental difference in the effect of cyanide and azide on respiration, it has been postulated that an alternative pathway, independent of the cytochrome-cytochrome oxidase system, accounts for the azide-insensitive resting respiration (2, 5). Thus far, however, no evidence has been presented as to the nature of this pathway. Horecker and Kornberg (7) have demonstrated that ferricytochrome c combines with cyanide ion under physiological conditions, and it became of interest to study the action of azide on this substance, since with the isolated compound the quantitative as well as the qualitative aspects of the reactions could be examined.

EXPERIMENTAL

Cytochrome c—This was prepared from calf heart by the recently modified method of Keilin and Hartree (8). The Fe content of this prepara-
tion as determined by the method of Delory (9) was 0.33 per cent. The cytochrome c solutions were acidified to pH 4.0 with 0.1 N HCl and aerated to insure complete oxidation before neutralization and addition of buffer for each experiment.

**Azide Solutions**—Azide solutions were prepared from Eimer and Amend sodium azide and diluted in 0.05 M phosphate buffer unless otherwise indicated.

**Succinic Dehydrogenase**—This was prepared from rat heart by grinding with sand in 2 volumes of m/15 Na₂HPO₄ and centrifuging lightly.

**Spectrophotometry**—The spectrophotometric measurements were made with the Beckman spectrophotometer, with a spectral band width which varied from 7 A in the blue to 30 A in the red and infra-red. The concentration of the cytochrome c solutions was determined at 5500 A after reduction with Na₂S₂O₄ and calculated with the extinction coefficient of 2.80 x 10⁴ (concentration in moles per liter). The values obtained were in agreement with those predicted from the Fe content. When concentrated solutions of sodium azide were prepared, these were found to have a slight yellow to red color and appropriate blank measurements were made to correct the absorption. All measurements were made in cells having a length of 10.0 mm.

**Absorption Spectra**

Azide was found to form a well defined complex with ferricytochrome c with a spectrum differing significantly from that of free cytochrome, as is shown in Figs. 1 and 2. The point of maximal absorption is shifted from 5300 to 5400 A, an even greater shift than is observed with the cyanide complex, and a small new band appears at about 5700 A. The band for ferricytochrome c at 6925 A² is absent in the azide complex, as it is in the cyanide complex, but below 6500 A the absorption is increased. The extinction coefficients for the azide complex were calculated from data obtained with solutions containing 0.16 and 0.67 M sodium azide, for reasons indicated in the following section.

**Dissociation Constant**

Two wave-lengths, 6950 and 6300 A, at which the azide complex differs greatly in absorption from the free cytochrome c, were used for the determination of the dissociation constant. Fig. 3 shows the effect of increasing concentration of azide on the calculated extinction coefficients. At 6950 A

¹ In a recent review Theorell (10) has confused this band with some previously reported by Bigwood, Thomas, and Wolters (11). These authors described bands at 6400 to 6450 A and at 6750 A, which were obtained with ferricytochrome c only in alkaline solution and which are evidently distinct from the band at 6925 A.
Fig. 1. Absorption spectra of cytochrome c and derivatives. Concentration, $3.2 \times 10^{-4}$ M; cell length, 10.0 mm.

Fig. 2. Absorption spectra of cytochrome c and derivatives in the red region. Concentration, $3.2 \times 10^{-4}$ M; cell length, 10.0 mm.
A there is a progressive decrease in absorption and at 6300 A a progressive increase, in both cases approaching asymptotic values which represent the extinction coefficients of the azide complex. However, in the very concentrated azide solutions required for complete formation of the complex, secondary effects, such as the formation of turbidity, interfered with the accurate determination of the extinction coefficients. These were obtained by a graphical method.

Fig. 3. Effect of azide on apparent extinction coefficients of ferricytochrome c. All solutions were diluted in 0.05 M phosphate buffer.

The rectangular hyperbolae shown in Fig. 3 may be represented by Equation 1,

\[ \epsilon_{\text{complex}} - \epsilon_{\text{observed}} = (\epsilon_{\text{complex}} - \epsilon_{\text{free}}) \cdot \frac{(N_3^-)}{K + (N_3^-)} \]

where \( \epsilon_{\text{complex}}, \epsilon_{\text{free}}, \) and \( \epsilon_{\text{observed}} \) are the extinction coefficients for the complex, the free ferricytochrome c, and the mixtures, respectively; \( (N_3^-) \) is the concentration of azide ion, and \( K \) the dissociation constant. From the reciprocal of Equation 1 a linear relation is obtained,

\[ \frac{1}{\epsilon_{\text{complex}} - \epsilon_{\text{observed}}} = \frac{1}{\epsilon_{\text{complex}} - \epsilon_{\text{free}}} \cdot \frac{1}{(N_3^-)} + \frac{1}{\epsilon_{\text{complex}} - \epsilon_{\text{free}}} \]

as is shown in Fig. 4. \( \Delta \epsilon \) denotes the change in extinction coefficient produced by addition of azide. From the intercept the extinction coefficient of the complex may be calculated and from the slope the value of
the dissociation constant. At the two wave-lengths, values for the dissociation constant of 0.16 and 0.14 were obtained.

In Fig. 5 the experimental points are fitted to the theoretical curve for a dissociation constant of 0.15, with 1 mole of azide ion assumed to combine with 1 of cytochrome c. Also shown are values calculated on the basis of the hydrazoic acid (HIN₃) concentration calculated from the hydrolysis of the azide ion, with a value for the dissociation constant of HN₃ of 1.9 × 10⁻⁶ (12). In the pH range from 5.9 to 7.8 the points calculated on the basis of azide ion are in agreement with the theoretical curve, while on the basis of hydrazoic acid the points at each pH form a distinct set. It is thus concluded that, as in the case of cyanide, ferricytochrome c combines with 1 mole of azide ion.

Reversibility

In order to establish that a true reversible equilibrium is reached when azide reacts with cytochrome c, the effect of succinic dehydrogenase on the complex was studied. At a concentration of N₃⁻ of 0.1 M, all of the cytochrome c is completely reduced in a few minutes, despite the fact that 40 per cent of the oxidized cytochrome is combined with azide. At somewhat higher azide concentrations the same general picture is observed,
although at concentrations high enough to bring about complete forma-
tion of complex the dehydrogenase is inhibited as well, so that no reduction
is observed. This inhibition of succinic dehydrogenase is apparently
similar to that observed by Potter (13) for chloride ion, and is obtained
with sodium chloride and sodium nitrate as well as with sodium azide.

Effect of Azide on Autoxidation of Ferrocytochrome c

Although azide did not produce any changes in the absorption spectrum
of ferrocytochrome c, it was found to accelerate the autoxidation of this

![Graph showing dissociation curve for azide-ferricytochrome c. The line represents the theoretical curve for a dissociation constant of 0.15, with 1 mole of azide assumed to combine with 1 mole of ferricytochrome c.](http://www.jbc.org/)

substance markedly, as is shown in Table I. This effect on the rate of
autoxidation was suggestive of the formation of an azide-ferrocytochrome
c complex, with a decreased oxidation-reduction potential, but was subse-
quently concluded to be due to the catalytic action of an iron-azide com-
plex formed from traces of inorganic iron. From Table I, it will be seen
that added iron has a promoting effect on the azide catalysis, while iron
in the absence of azide is without effect.

The ferric-azide complex is responsible for the color of concentrated
azide solutions previously mentioned, and exhibits an absorption maximum
at 4250 Å. On reduction the color and absorption band disappear. From
the change in absorption at 4250 and 5500 Å, it was established that there
is an instantaneous and quantitative reaction between the ferric-azide complex and ferrocytochrome c, following which, in the presence of air, the iron-azide complex is reoxidized; the rate of this step determines the over-all rate of ferrocytochrome c oxidation. Thus the alternate reduction and oxidation of the iron-azide complex brings about the complete oxidation of ferrocytochrome c, even though the iron is present in only minute quantities.

**Table I**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>NaN₃ mole per l.</th>
<th>Fe γ per cc.</th>
<th>Rate of autoxidation k' × 10⁻¹⁰</th>
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<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0</td>
<td>9</td>
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<tr>
<td></td>
<td>0.33</td>
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<td></td>
<td>1.00</td>
<td>0</td>
<td>890</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
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<td></td>
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</tr>
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<td>0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.33</td>
<td>15</td>
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</tbody>
</table>

*First order reaction velocity constant as defined in the succeeding paper.

**Table II**

<table>
<thead>
<tr>
<th>Dissociation constants at 25°, mole per l.</th>
<th>Azide</th>
<th>Cyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ferrocytochrome c as complex at equilibrium at 25°, pH 7.4 in 0.001 M KCN or NaN₃</td>
<td>0.15</td>
<td>2 × 10⁻⁴</td>
</tr>
<tr>
<td>Rate of formation</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td>pH effect</td>
<td>Absent</td>
<td>Present</td>
</tr>
</tbody>
</table>

**Discussion**

In Table II the properties of the azide complex are compared with those of the cyanide complex as previously reported (7). While cyanide forms a stable complex with a dissociation constant of 2 × 10⁻⁴, the rate of formation is slow; only half the cytochrome c is combined after 60 minutes in 0.001 M KCN at 25°, pH 7.4. On the other hand, the azide complex is much less stable, with a dissociation constant of about 0.15, but equilibrium is reached within the 30 seconds required for the spectrophotometer reading.
It is interesting to note the fraction of the ferricytochrome $c$ which is combined at pH 7.4 in 0.001 M inhibitor at 25°, since these conditions are frequently used in studies in vivo. While formation of the cyanide complex at equilibrium is almost complete, 87 per cent, practically none of the ferricytochrome $c$ would combine with azide.

The effect of pH on the degree of complex formation in azide and in cyanide is to be compared with the pH effect observed on the action of these inhibitors in vivo. The pH effect is absent in the range from 5 to 8 in the case of azide and present in the case of cyanide, indicating again that it is the ionic form of these substances which combines with the cytochrome $c$. Thus in the case of azide the concentration of the ionic form does not change appreciably with pH, since even at the acid end of the range practically all of the salt is ionized. In the case of cyanide, however, hydrolysis in the pH range studied is practically complete; in this case variations in pH produce large changes in the concentration of cyanide ion. These observations are not in accord with the pH effect observed in vivo, as is discussed in the succeeding paper.

The effect of azide on the oxidation of ferrocytochrome $c$ suggests the possibility of a similar catalysis occurring in studies of the azide inhibition of respiration. To what extent this catalytic mechanism is able to replace the cytochrome oxidase system remains to be determined, but it should be considered whenever the inhibition of respiration by azide is incomplete.

It has been established that the affinity of cytochrome $c$ for azide is very much less than for cyanide. Thus an alternative pathway of respiration might involve an iron-containing catalyst with similar affinities. In order for cytochrome $c$ itself to account for the differential effects of azide and cyanide, it becomes necessary to introduce a cyanide- and azide-insensitive mechanism for the oxidation of ferrocytochrome $c$. The shift from resting to activity respiration would then involve an activation of the powerful cytochrome oxidase system, as has previously been suggested (2, 5). The activity respiration would thus be sensitive to both cyanide and azide, while only cyanide by combining with ferricytochrome $c$ would affect the resting respiration. Experimental verification of this hypothesis must await the demonstration of an oxidative enzyme with the required characteristics.

SUMMARY

1. A complex is formed between ferricytochrome $c$ and azide ion which contains 1 mole of azide ion per mole of cytochrome $c$. The complex is rapidly formed, but much less stable than the cyanide complex.

2. The absorption maximum is shifted from 5300 to 5400 \(\mu\) on formation of the complex and marked changes in absorption are observed in the red region.
3. The dissociation constant as determined spectrophotometrically is 0.15 at 25°. The formation of the complex is reversible.
4. The properties of the azide complex are compared with those of the cyanide complex and the possible physiological significance is discussed.

BIBLIOGRAPHY

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