The Effect of Deoxycholate on Cytochrome Oxidase*

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Sodium deoxycholate and sodium cholate have been widely used to solubilize mitochondrial enzymes ever since their use by Keilin and Hartree for clarifying heart muscle preparations (1), by Hopkins, Lutwak-Mann, and Morgan (2) for extracting succinic dehydrogenase, by von Euler and Hellstrom (3) for preparing cytochrome b and cytochrome oxidase (cytochrome c:O₂ oxidoreductase, EC 1.9.3.1), and by Yakushiji and Okumiki (4) for preparing cytochrome c₁. The first highly active soluble form of cytochrome oxidase was obtained with the aid of deoxycholate in 1947 (5, 6), and since that time many investigators have used both deoxycholate (e.g.7-10) and cholate (e.g.8-13) to extract cytochrome b and cytochrome c₁ as well as cytochrome oxidase.

The ability of the bile salts to solubilize the cytochromes is complicated by the fact that these reagents may also modify enzyme activity; under certain conditions both deoxycholate and cholate activate cytochrome oxidase (e.g.11, 14-19), whereas under other conditions they are powerful inhibitors (e.g.8, 11, 15-24). Nevertheless, despite their widespread use, few attempts have been made to determine the mechanism of these effects. In 1955 Smith (16) suggested, on the basis of spectrophotometric studies, that cholate probably inhibits cytochrome oxidase by interfering with its reduction by cytochrome c rather than its reoxidation by oxygen. On the other hand, Martin and Stole (23) reported in 1960 that both reduction and reoxidation of cytochrome oxidase were inhibited by cholate, although the combination of cytochrome oxidase with oxygen was not affected. To our knowledge, similar studies with deoxycholate have not been reported.

Our own work on this problem stems from the observation that incubation of a deoxycholate-solubilized preparation of cytochrome oxidase for 50 minutes at 37° markedly inactivated the enzyme. Subsequent studies showed that the inactivation was caused by the deoxycholate present in the cytochrome oxidase preparation; the bile salt apparently converted some of the cytochrome oxidase to a denatured form which was not reducible by its natural substrate, reduced cytochrome c. Furthermore, the spectrum obtained after reduction of the denatured enzyme with dithionite differed from that of the normal enzyme. Incubation of reduced cytochrome oxidase under the same conditions did not modify the spectrum, nor did it prevent reoxidation of the enzyme by oxygen.

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EXPERIMENTAL PROCEDURE

Cytochrome oxidase was prepared by a modification (25) of the method of Wainio et al. (6). The insoluble heart muscle particles were extracted with 2% deoxycholate and centrifuged at 28,700 × g for 1 hour; the precipitate was then extracted with 3% deoxycholate and the supernatant obtained after centrifugation was used. This is referred to as the "2 to 3% preparation." For some experiments, this preparation was dialyzed and treated with alumina C₇ gel and ammonium sulfate in order to remove some of the deoxycholate (25); this preparation is designated the "ammonium sulfate preparation." Both the 2 to 3% and ammonium sulfate preparations were lyophilized, and the dried powder was dissolved in 0.1 M Na₂HPO₄-KH₂PO₄ buffer, pH 7.1, just before use.

The deoxycholate content of the preparations was determined by a micro modification of the method of Szalkowski and Mader (26). A 10- to 20-μl sample was pipetted into a microtube and evaporated to dryness in an oven. The precipitate was suspended in 1 ml of 95% ethanol, the mixture was centrifuged for 10 minutes at 1000 × g, and 250 μl of the supernatant were transferred to another microtube. After this was evaporated to dryness, 60 μl of the salicylaldehyde-sulfuric acid reagent were added and the tubes were placed in a 40° water bath for 15 minutes. The tubes were allowed to cool at room temperature for 5 minutes and 400 μl of glacial acetic acid were added. The contents of the tubes were mixed for 10 seconds, the mixture was permitted to stand at room temperature for 5 minutes, and the optical density was read in a Beckman model DU spectrophotometer at 680 mμ.

Sodium deoxycholate was used as purchased from the Fisher Scientific Company. Cytochrome c, type II, was purchased from the Sigma Chemical Company and was dissolved in water to the desired concentration.

Spectra were recorded with a Cary model 11 recording spectrophotometer. The instrument was adapted for use with small volumes by inserting in the slot in the cuvette holder a diaphragm containing a pinhole. In all experiments the cuvettes contained 2 ml of the cytochrome oxidase preparation at a concentration of 5 mg (dry weight) per ml; this is equivalent to about 0.5 mg of protein per ml in the 2 to 3% preparation or about 0.25 mg of protein per ml in the ammonium sulfate preparation. Where noted, the preparation was reduced by adding a few grains of sodium dithionite. Since we have confirmed previous reports (27, 28) that reduction of cytochrome oxidase and other hematin...
a compounds by dithionite requires several minutes, spectra were routinely recorded immediately upon addition of dithionite, again approximately 5 minutes later, and finally at the end of 30 minutes. In most cases reduction was complete within the initial 5-minute period.

Reduction of cytochrome oxidase by ascorbic acid and cytochrome c was studied in the modified form (29) of the anaerobic spectrophotometer cell designed by Lazorow and Cooperstein (30). Cytochrome oxidase (2 ml) and cytochrome c (0.1 ml; 15 μg) were pipetted into the main body, and 0.2 ml of a neutralized 0.115 M solution of ascorbic acid was placed in the side arm. The cell was flushed for 10 minutes with nitrogen which had been passed through electrically heated copper screens to remove traces of oxygen. After a zero time spectrum was recorded, the contents of the side arm were tipped into the main body, and spectra were recorded at approximately 1-minute intervals. In all experiments spectra were recorded for at least 30 minutes although in most cases reduction was complete within 10 to 15 minutes.

Cytochrome oxidase activity was determined manometrically with the hydroquinone system previously described (31); in all experiments 1 ml of the enzyme sample was used.

The pyridine-hemochromogen of cytochrome oxidase was prepared by the method described by Green, Mi, and Kohout (32); 0.2 ml of 1 M NaOH and 2.2 ml of pyridine were added to 2 ml of the cytochrome oxidase preparation and the mixture was allowed to stand for 15 minutes at room temperature. After centrifugation the spectrum of the supernatant was recorded.

RESULTS

Effect of Incubating Cytochrome Oxidase at 37°—As can be seen in Table I, when a 2 to 3% preparation of cytochrome oxidase was incubated for 30 minutes at 37°, there was marked loss in activity, and this inactivation was greater in the more concentrated solutions.1 Fig. 1 demonstrates that in the same period the amount of enzyme which could be reduced by a mixture of ascorbic acid and cytochrome c also decreased; this is indicated by the absence of a peak at 600 mμ and the presence of only a slight shoulder at 441 mμ. Furthermore, whereas the oxidized spectrum of the incubated preparation was essentially the same as that of the control, the normal reduced spectrum of cytochrome oxidase failed to appear after addition of dithionite to the incubated sample (Fig. 2); the Soret peak was at 429 mμ, with only a slight skewness toward 442 mμ, and the usual 601 mμ peak was almost completely absent. Subsequent aeration of the incubated preparation restored the normal oxidized spectrum.

Effect of Time and Temperature of Incubation—The degree of inactivation of cytochrome oxidase depended upon the time and temperature of the incubation period. At 37° there was little or no effect after 5 minutes of incubation, but at the end of 10 minutes the spectrum obtained on addition of dithionite showed considerable flattening of both the 600 mμ and 442 mμ peaks (Fig. 3). After 30 minutes of incubation there was almost no 600 mμ peak and in the Soret region there was a plateau with a slight peak at 429 mμ; these changes were more pronounced after 45 minutes. At the same time, the total amount of cytochrome oxidase which could be reduced by a mixture of ascorbic acid and cytochrome c also progressively decreased, although there was little or no change in the initial rate of reduction (Fig. 4).

When the incubation was carried out at room temperature the inactivation was considerably slower; at this temperature, no change was observed for 2 hours, but by the next day (28 hours) the loss of the 600 mμ peak and the shift of the Soret peak to 429 mμ after addition of dithionite were quite evident. Similarly, after 3 hours incubation at room temperature cytochrome oxidase was reduced normally by a mixture of ascorbic acid and cytochrome c, but no reduction was observed after the sample had been incubated for 28 hours.

When the incubation was carried out at 0°, a still longer period was necessary before any changes became evident. Even after 10 days there was only a barely detectable shift in the position of the Soret peak after reduction by dithionite. However, by the second day a marked decrease in the amount of enzyme which could be reduced by a mixture of ascorbic acid and cytochrome c was apparent; this is indicated in Table II by the smaller increase in extinction at 442 mμ after reduction was complete. The fact that the amount of enzyme which could be reduced by ascorbic acid plus cytochrome c decreased before any change in the spectrum after reduction by dithionite was evident is not surprising, because enzymatic reduction is a more sensitive indicator of damage than is chemical reduction.

Effect of Deoxycholate Concentration—Since the changes noted above were more evident when higher concentrations of the enzyme were incubated, it was thought that they might be due to the deoxycholate present in the preparation. Therefore, these experiments were repeated with the ammonium sulfate preparation, which, as stated previously, contains a lower concentration of the bile salt. Fig. 5 shows that when this preparation was incubated at a concentration of 5 mg per ml (deoxycholate concentration, 1.07 mg per ml) the normal reduced spectrum was obtained after addition of dithionite. On the other hand, when graded amounts of deoxycholate were added before incubation (Fig. 5), the spectra obtained after addition of dithionite gradually assumed the abnormal form observed with the 2 to 3% preparation. Similarly, the cytochrome oxidase in an ammonium sulfate preparation was readily reduced by a mixture of ascorbic acid and cytochrome c even after incubation for 30 minutes at 37°, but when more deoxycholate was added before

1 We have previously reported (33) no loss in activity on incubating a deoxycholate-solubilized preparation of cytochrome oxidase at 37° for 30 minutes; however, far lower concentrations of the enzyme were used in the previous experiments than in those reported in Table I.
Effect of Incubation of Reduced Cytochrome Oxidase—Incubation of oxidized cytochrome oxidase under anaerobic conditions did not protect it against the action of deoxycholate; however, when incubation, the amount of enzyme which could be reduced decreased (Table III).

It should be noted that the concentration of deoxycholate which had to be added to the ammonium sulfate preparation in order to produce the same degree of spectral alteration observed with the 2 to 3% preparation was far greater than the concentration of deoxycholate present in the latter preparation (3.22 mg per ml). This suggests that either (a) the deoxycholate already present in the cytochrome oxidase preparation is more effective than that added just before incubation, (b) the 2 to 3% preparation is more sensitive to deoxycholate than is the ammonium sulfate preparation, or (c) the observed effects are due to the combined action of deoxycholate and some other factor present in higher concentration in the 2 to 3% preparation.

Effect of Incubation of Reduced Cytochrome Oxidase—Incubation of oxidized cytochrome oxidase under anaerobic conditions did not protect it against the action of deoxycholate; however, when


Figure 5. Effect of added deoxycholate on the spectrum of cytochrome oxidase. Cytochrome oxidase (ammonium sulfate preparation) was incubated at a concentration of 5 mg per ml for 30 minutes at 37°. The deoxycholate concentration in the preparation was 1.97 mg per ml; deoxycholate was added before incubation to bring the concentrations to those indicated. The curves shown were recorded at least 30 minutes after addition of dithionite. The curve of the unincubated control is not shown; it was identical with that observed after incubating the preparation in 1.97 mg of deoxycholate per ml. The extinction at 650 μm was arbitrarily set at the same point for all curves.

Table III

Effect of deoxycholate on reduction of cytochrome oxidase by ascorbic acid + cytochrome c

<table>
<thead>
<tr>
<th>Deoxycholate concentration (mg/ml)</th>
<th>Total absorbancy change at 442 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.26, unincubated</td>
<td>0.074</td>
</tr>
<tr>
<td>1.26</td>
<td>0.086</td>
</tr>
<tr>
<td>2.25</td>
<td>0.064</td>
</tr>
<tr>
<td>5.0</td>
<td>0.042</td>
</tr>
<tr>
<td>7.5</td>
<td>0.037</td>
</tr>
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</table>

The enzyme was first reduced by dithionite and then incubated anaerobically, the normal reduced spectrum was observed (Fig. 6). (The small differences between the spectrum of the incubated sample and that obtained before incubation are due to clouding which occurred during incubation.) Furthermore, after aeration the enzyme was rapidly oxidized. When more dithionite was added to this sample after aeration there was considerable cloudiness, which made interpretation of the spectrum difficult; however, as seen in Fig. 6, the Soret peak was at 431 μm, suggesting that the reduced enzyme may have become modified during incubation but that this only became evident after oxidation and subsequent reduction by dithionite.

When this experiment was carried out with ascorbic acid plus cytochrome c as the reducing agent instead of dithionite (Fig. 7), the spectrum obtained after incubation showed the presence of some reduced cytochrome oxidase; however, the amount was

Figure 6. Effect of incubation on cytochrome oxidase previously reduced by dithionite. The concentration of cytochrome oxidase (ammonium sulfate preparation) was 5 mg per ml. Deoxycholate was added to a final concentration of 7.5 mg per ml. Incubation period was 30 minutes at 37°. The extinction at 650 μm was arbitrarily set at the same point for all curves. Curve 1, oxidized, before incubation; 2, reduced, before incubation; 3, incubated; 4, aerated; 5, 20 minutes after second addition of dithionite.

Figure 7. Effect of incubation on cytochrome oxidase previously reduced by ascorbic acid + cytochrome c. The concentration of cytochrome oxidase (ammonium sulfate preparation) was 5 mg per ml. Deoxycholate was added to a final concentration of 7.5 mg per ml. Incubation period was 30 minutes at 37°. The ascorbic acid concentration was twice that usually used. The extinction at 650 μm was arbitrarily set at the same point for all curves. Curve 1, reduced, before incubation; 2, incubated; 3, aerated; 4, 30 minutes after second addition of ascorbic acid.
far less than before incubation. When the mixture was aerated, the normal spectrum of oxidized cytochrome oxidase reappeared. The decrease in the amount of reduced cytochrome oxidase which occurred during incubation may have resulted from denaturation of the enzyme during the incubation period; however, it seems more likely that during incubation the ascorbic acid became depleted and cytochrome oxidase was gradually oxidized by traces of oxygen in the cuvette, since addition of a second portion of ascorbic acid and restoration of anaerobic conditions (Fig. 7, Curve 4) resulted in reduction of the enzyme essentially to the original level (judged by the height of the 441 mp peak relative to the 415 mp peak of reduced cytochrome c).

It should be noted that the amount of cytochrome oxidase which was reduced by the second addition of ascorbic acid varied in different experiments, and only occasionally did it reach the original level as shown in Fig. 7; however, since this experiment is technically difficult and many extraneous factors could prevent complete reduction, it seems likely that the experiment shown in Fig. 7 represents the true situation.

**Effect of Deoxycholate on Cytochrome Oxidase**

In an attempt to determine whether or not deoxycholate affects the heme moiety itself, the pyridine-hemochromogens obtained from the incubated and unincubated preparations were compared. As can be seen in Fig. 8, essentially no difference was found. Furthermore, no heme was detected in a trichloroacetic acid extract of either the incubated or unincubated preparation, indicating that the changes observed after incubation with deoxycholate did not result from detachment of the heme from the protein.

**Effect of Cholate on Cytochrome Oxidase**

Our finding that incubation of cytochrome oxidase with either deoxycholate or cholate prevented subsequent reduction of the enzyme by reduced cytochrome c is consistent with the previous report of Smith (16), who found that in a system containing p-phenylenediamine, cytochrome c, and cytochrome oxidase, cytochrome c was nearly completely reduced in the steady state when high cholate concentrations were present. In our experiments (Fig. 4), the denatured cytochrome oxidase formed by the action of deoxycholate did not decrease the rate of reduction of the remaining unmodified enzyme, i.e., it did not act as a competitive inhibitor; therefore the bile salts probably modify the protein structure in such a way that cytochrome oxidase loses its ability to bind its natural substrate, reduced cytochrome c. On the other hand, in contrast to the report of Martin and Stotz, who used cholate (23), we found no evidence that deoxycholate interferes with the oxidation of reduced cytochrome oxidase. In our experiments a sample of cytochrome oxidase which had been incubated with deoxycholate and then reduced by dithionite was readily oxidized by aeration. Furthermore, incubation of cytochrome oxidase with deoxycholate after prior reduction of the enzyme with either dithionite or ascorbic acid

**DISCUSSION**

<table>
<thead>
<tr>
<th>Cholate concentration (mg/ml)</th>
<th>Total absorbancy change at 442 mp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.061</td>
</tr>
<tr>
<td>5</td>
<td>0.040</td>
</tr>
<tr>
<td>10</td>
<td>0.031</td>
</tr>
<tr>
<td>20</td>
<td>0.025</td>
</tr>
<tr>
<td>30</td>
<td>0.020</td>
</tr>
<tr>
<td>50</td>
<td>0.005</td>
</tr>
<tr>
<td>7.5 mg of deoxycholate per ml; no cholate added</td>
<td>0.019</td>
</tr>
</tbody>
</table>
indicating that essentially the same amount of oxidized cytochrome oxidase was taken with the reduced enzyme present in the control is indicated by the same amount of reduced cytochrome oxidase that disappeared in both the control and incubated sample (reduced with dithionite) was stored for 2 to 3 days at 0-5°C, there was a decrease in the ratio of the extinction at 605 μm to that at 630 μm after reduction; this was accompanied by decreased absorption at 444 μm and an increase at 425 μm. Although we did not observe this change at 0°C, the changes which we noted at higher temperatures showed the same pattern; therefore it seems likely that their results were also due to the presence of the bile salts. Similarly, the shifts in the reduced peaks observed by Smith (16) when a cytochrome oxidase preparation was treated with 4% cholate probably resulted from the same phenomenon. On the other hand, the spectral changes reported by Elliot, Hulsmann, and Slater (34) and Morrison and Stotz (35) differ from our findings in several ways and therefore appear to involve a different mechanism.

The abnormal spectrum observed after addition of dithionite to an incubated sample of a deoxycholate-solubilized cytochrome oxidase preparation failed to produce the normal spectrum of reduced cytochrome oxidase in agreement with an earlier report of Griffiths and Wharton (8). These investigators found that when their cytochrome oxidase preparation (made with cholate and deoxycholate) was stored for 2 to 3 days at 0-5°C, there was a decrease in the ratio of the extinction at 605 μm to that at 630 μm after reduction; this was accompanied by decreased absorption at 444 μm and an increase at 425 μm. Although we did not observe this change at 0°C, the changes which we noted at higher temperatures showed the same pattern; therefore it seems likely that their results were also due to the presence of the bile salts. Similarly, the shifts in the reduced peaks observed by Smith (16) when a cytochrome oxidase preparation was treated with 4% cholate probably resulted from the same phenomenon. On the other hand, the spectral changes reported by Elliot, Hulsmann, and Slater (34) and Morrison and Stotz (35) differ from our findings in several ways and therefore appear to involve a different mechanism.

The abnormal spectrum observed after addition of dithionite to an incubated sample of cytochrome oxidase could be due to failure of this reagent to reduce the enzyme completely (8); however, examination of the difference spectra of the incubated and unincubated preparations (Fig. 10) argues against this hypothesis. Whereas the 444 μm peak in the difference spectrum (reduced minus oxidized) of the incubated enzyme was only about two-thirds as high as in the control, indicating that less reduced cytochrome oxidase had been formed (Fig. 10a), there was very little difference in the depth of the trough at 411 μm, indicating that essentially the same amount of oxidized cytochrome oxidase had disappeared in both the control and incubated samples. This is more clearly seen in Fig. 10b, which shows the curve obtained when the spectrum of the unincubated sample (reduced with dithionite) was taken with the reduced incubated sample as the reference; the greater amount of reduced cytochrome oxidase present in the control is indicated by the 444 μm peak in this curve, whereas no corresponding trough in the region of the peak of oxidized cytochrome oxidase can be seen. These results suggest that cytochrome oxidase was completely reduced by dithionite but that part of it had been modified so that the Soret peak of the reduced form was shifted towards the blue. This is supported by the curve obtained when the spectrum of the reduced incubated enzyme was measured using as the reference a control sample which had been diluted to the extent necessary to eliminate the difference between the two samples at 444 μm; in this way the contribution of reduced cytochrome oxidase to the spectrum of the incubated preparation was eliminated. The resulting curve (Fig. 10b) clearly showed a peak centered about 427 μm. (The presence of this component in the incubated sample is also suggested by a slight asymmetry at about 430 μm evident in the reduced minus oxidized difference spectrum of the incubated enzyme, but this is not apparent in Fig. 10a because of the small scale.) This peak presumably belongs to a denatured form of cytochrome oxidase in which either the heme moiety or the linkage of the heme to the protein has been modified sufficiently to cause a change in the spectrum of the reduced form; since our experiments failed to show any evidence of a change in the heme itself, the latter appears to be the more likely explanation. The 429 μm peak observed in the reduced spectrum of the incubated preparation (Fig. 2) probably results from a mixture of this denatured enzyme with its peak at 427 μm and the normal form with its peak at 442 μm.

These results are of particular importance in connection with the use of bile salts for extracting cytochrome b. Since there is no well established catalytic assay for cytochrome b, preparations of this enzyme have usually been characterized only by spectral analyses (10, 31). The Soret peak of reduced cytochrome b is at about 430 μm (1, 10, 12, 31); therefore, many cytochrome b preparations may consist, at least partially, of a deoxycholate-solubilized cytochrome oxidase preparation which could show a Soret peak at about 430 μm which could easily be ascribed to cytochrome b. Therefore, extreme caution is indicated in drawing any conclusions from preparations made with bile salts.

**SUMMARY**

Incubation of cytochrome oxidase with either deoxycholate or cholate produced marked loss in activity which was associated with a decrease in the amount of enzyme which could be reduced by reduced cytochrome c. The amount of inactivation increased with increased concentration of the bile salts and with
increased time and temperature of incubation. Incubation of reduced cytochrome oxidase with deoxycholate did not interfere with its reoxidation by oxygen.

Incubation of oxidized cytochrome oxidase with the bile salts did not modify its spectrum, but after reduction of the incubated preparation with dithionite the 691 nm peak was lower than in the control and the Soret peak was at 429 nm instead of 442 nm. The abnormal position of the Soret peak probably is due to the presence, after incubation, of a mixture of normal reduced cytochrome oxidase and a denatured form of the enzyme with a peak at 427 nm in the reduced state.

REFERENCES
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