The Respiratory Chain in Phosphorylating Subfragments of Mitochondria Prepared with Digitonin*

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Previous communications from this laboratory (1-6) have described various enzymatic reactions associated with oxidative phosphorylation in subfragments of rat liver mitochondria obtained by treatment with digitonin. This paper describes the electron transport system of such mitochondrial fragments.

Although some chemical and physical treatments affecting the morphology of mitochondria have been observed to cause substantial changes in the molar ratios of the various respiratory carriers (7-9), the results reported in this paper show that mitochondrial subfragments obtained by treatment of rat liver mitochondria with digitonin contain complete and substantially intact respiratory chains. With the exception of pyridine nucleotide, the relative molar concentrations of the carriers in the fragments, in comparison with those in intact mitochondria, is not significantly altered by the digitonin treatment. The occurrence of a pyridine nucleotide transhydrogenase in the subfragments, in comparison with those in intact mitochondria, is not sufficient to cause significant shifts in absorption maxima. The occurrence of pyridine nucleotide transhydrogenase in the subfragments is also demonstrated.

EXPERIMENTAL

Methods—The mitochondrial subfragments were prepared and assayed for oxidative phosphorylation as described previously (10, 1) and only those preparations manifesting maximal phosphorylating efficiency were employed. Difference spectra were measured on the expanded per cent transmission scales of the Beckman DK-2 ratio recording spectrophotometer and were replotted in terms of optical density. Due to the turbidity of the preparations, control experiments were performed to eliminate errors due to light scattering and the effects of stray light (11). Changes in light scattering caused by dilution during the course of the spectrophotometric experiments were kept to a minimum by adding reagents in relatively small volumes (from 1 to 5 μl.). To test for possible shifts in the position of the absorption maxima or minima due to light scattering, the spectrum of a dilute solution of Fe+++ cytochrome c was recorded between 380 and 630 nm with the mitochondrial subfragments present in both the reference and sample cuvettes. There were no significant differences between this spectrum and one of oxidized cytochromes c, a, and a₃, and flavoprotein (7). Upon the addition of cyanide to the anaerobic, fully reduced system, which remained constant until all of the dissolved oxygen has been utilized, whereupon the carriers become fully reduced to produce the difference spectrum of Curve B. Typical peaks and depressions were observed in the completely reduced spectrum of Curve B which correspond to the reduced forms of cytochromes c, a, and a₃, and flavoprotein (7). Upon the addition of cyanide to the anaerobic, fully reduced system, the only change in the spectrum was a decrease in the 445 nm peak, due to the formation of the cyanide complex of reduced cytochrome a₃. This spectrum does not demonstrate unequivocally the presence of a pyridine nucleotide transhydrogenase in the subfragments, in comparison with those in intact mitochondria. This paper describes the electron transport system of such mitochondrial fragments.

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readily permitted the visualization of the bands of reduced cyto-
mycin A to block reoxidation of cytochrome b, the LY-, p-, a, and P-hydroxybutyrate by the digitonin particle (1, 10). With anti-
chondrial electron transport system at the level of cytochrome b
shown as Curve B in Fig. 2. Antimycin A inhibits the mito-
chondrial respiratory enzymes in the aerobic steady state and that
of the antimycin A inhibited system, which indicates that cyto-
chrome b is apparently the only measurably reduced cytochrome
in the aerobic steady state condition. With the \( \Delta \text{OD}_{560} \) (13) as a measure of reduced cytochrome b, and since it may be
assumed that cytochrome b is completely oxidized in the reference
cell (13), it was determined from a number of such experiments
with the digitonin preparation that cytochrome b is approxi-
mately 45 per cent reduced in this aerobic steady state. For
comparison, Chance and Williams (13) have found that cyto-
chrome b is reduced about 16 per cent in intact rat liver mito-
chondria under similar conditions, i.e., “state 3” (high concen-
tration of ADP and substrate) minus “state 2” (high ADP, no
substrate).

Concentration and Molar Ratios of Respiratory Carriers—In
Table I is a summary of the average concentration of the respira-
tory carriers in the electron transport system of four different
digonin preparations. There was very little variation between
preparations. Cytochromes \( a_0 \), \( a_1 \), and \( c \) were measured
in the totally reduced system as in Fig. 1; cytochrome b and flavo-
protein were determined in the antimycin A inhibited system,
which permits the determination of these two carriers without
interference from the other cytochromes. As can be seen from
a comparison of the last two columns, the molar ratios of the
cytochromes and flavoprotein in the digitonin preparation closely

![Fig. 1. Difference spectra of mitochondrial subfragments. The sample cuvette contained 0.01 M \( n^-(-) \beta \)-hydroxybutyrate, 0.0024 M ADP, 0.01 M inorganic phosphate (pH 6.5) and mitochondrial subfragments (100 \( \mu \)g N/ml.) in a total volume of 3.0 ml. The reference cuvette contained the same medium without \( n^-(-) \beta \)-hydroxybutyrate, representing the oxidized complex. In Curve A (broken line), the difference spectrum is due to changes in the oxidation-reduction concentrations of the respiratory enzymes upon initiation of rapid respiration with \( n^-(-) \beta \)-hydroxybutyrate, minus the nonrespiring reference sample containing the carriers in the fully oxidized state. This spectrum remains unchanged until all of the dissolved oxygen is utilized. Curve B is caused by the ensuing anaerobiosis of the sample cuvette which gives the difference between the spectrum of the enzymatically fully reduced carriers and the spectrum of the oxidized carriers. The roman numerals indicate the peaks corresponding to the reduced forms of carriers as follows: I, y-peak of reduced ferrocytochrome c; II, y-peak of reduced cytochrome \( a_0 \) and \( a_1 \); III, reduced flavoprotein; IV, y-peak of reduced cytochromes b and c; V, y-peak of reduced cytochrome c; VI, y-peak of reduced cytochromes a and \( a_1 \).

No distinct peaks for reduced cytochrome \( h \) were observed in
the anaerobic spectrum; the 430 and 563 \( \text{m} \mu \) peaks of reduced cytochrome \( b \) are presumably obscured by the \( y \) peaks of re-
duced cytochromes \( a_0 \), \( a_1 \), and \( a_2 \) and the \( x \) peak of reduced cyto-
chrome \( c \), respectively. In intact mitochondria, Chance and
Williams (7) were unable to observe peaks for reduced cyto-
chrome \( b \) in a difference spectrum of the fully reduced (state 5)
minus the aerobic steady-state (state 3) (in which cytochrome b
was reduced only 16 per cent), for similar reasons. However,
as is shown below, use of the respiratory inhibitor antimycin A
readily permitted the visualization of the bands of reduced cyto-
ochrome \( b \) in the digitonin fragments, as was the case in intact
mitochondria (7).

Aerobic steady-state and Effect of Antimycin A—The aerobic
steady-state condition of the respiratory pigments is again re-
produced in Curve A of Fig. 2. A higher enzyme concentration
was used in this experiment. The addition of 0.18 \( \mu \)g of anti-
mycin A per ml. to the sample cuvette produced the spectrum
shown as Curve B in Fig. 2. Antimycin A inhibits the mito-
chondrial electron transport system at the level of cytochrome b
(7) and has been shown to inhibit completely the oxidation of
\( \beta \)-hydroxybutyrate by the digitonin particle (1, 10). With anti-
mycin A to block reoxidation of cytochrome b, the \( a_0 \), \( b_0 \), and \( y \)-peaks of reduced cytochrome \( b \) at 563, 530 and 430 \( \text{m} \mu \), re-
spectively, and the depression at 460 to 465 \( \text{m} \mu \) caused by re-
duced flavoprotein are now quite clearly observed, with no in-
terference from the reduced bands of cytochromes \( c_1 \), \( a_0 \), and \( a_2 \),
which are all fully oxidized in this system. Thus the respiratory
chain of the digitonin subfragments contains functional cyto-
ochrome \( b \), as well as cytochromes c, a, and \( a_2 \).

A similarity can be observed between the difference spectrum of
the respiratory enzymes in the aerobic steady state and that
of the antimycin A inhibited system, which indicates that cyto-
ochrome \( b \) is apparently the only measurably reduced cytochrome
in the aerobic steady state condition. With the \( \Delta \text{OD}_{560} \) (13) as a measure of reduced cytochrome \( b \), and since it may be
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a comparison of the last two columns, the molar ratios of the
cytochromes and flavoprotein in the digitonin preparation closely

![Fig. 2. Difference spectra of the aerobic steady state and the effect of antimycin A. Sample cuvette contained 0.01 M \( n^-(-) \beta \)-hydroxybutyrate, 0.0024 M ADP, 0.01 M inorganic phosphate (pH 6.5) and mitochondrial subfragments (220 \( \mu \)g N/ml.) in a total volume of 3.0 ml. The reference cuvette contained the same medium without \( n^-(-) \beta \)-hydroxybutyrate, representing the oxidized complex. Curve A (broken line) represents the partially reduced cytochrome b, as was the case in intact mitochondria (7). Curve B (solid line) represents the proteins.
Ratios based on cytochrome a = 1.

Agreement with the values found by Chance and Williams (7) for intact rat liver mitochondria.

Numerous unsuccessful attempts have been made to measure spectrophotometrically the bound DPN of the digitonin preparation (17). No peak occurs at 340 μm in the aerobic steady state or in anaerobiosis; increasing the concentration of the enzyme complex, with subsequent increase in turbidity of the sample, places 340 μm beyond the useful range of the instrument, since at this wave length light scattering effects are severe. However, measurements of the bound DPN were carried out with a catalytic test employing diaphorase, after extraction of DPN from the multienzyme complex, and revealed the presence of about 0.3 mole of DPN per mole of cytochrome a. The amount of bound DPN in the mitochondrial subfragments is thus very small in contrast to intact rat liver mitochondria, where the total concentration of pyridine nucleotide is frequently 30 to 40 times that of the cytochrome a (7).

The total iron content of four different preparations was also determined, and has been found to be 35 to 45 μmoles of iron per mg. of N, or 5 to 7 times higher than can be accounted for by the known cytochromes present.

Reduction of Carriers with Hydrosulfite—Addition of hydrosulfite to systems which were already fully reduced enzymatically with β-hydroxybutyrate caused a new peak to appear at 430 μm, which obscured the γ-peak of cytochrome c, and produced a shoulder at 550 μm on the α-peak of cytochrome c. These observations suggested that a heme compound of unknown origin was present in the subfragments which was not reducible by β-hydroxybutyrate.

In Fig. 3 is shown the difference spectrum of the unidentified pigment, without interference from the functional cytochromes. In this experiment both the blank and sample cuvettes contained phosphate, ADP, β-hydroxybutyrate, and cyanide to reduce fully the enzymatically functional respiratory pigments. Hydrosulfite was now added to the sample cuvette, to give a difference spectrum of the unidentified pigment with peaks at 426, 530, and 556 μm. As can be seen from Fig. 3, there were no peaks which could be attributed to a further reduction of cytochromes c, a, or a3, indicating that these cytochromes are completely reduced by β-hydroxybutyrate.

Three possibilities have been eliminated as the source of this spectrum: (a) the peaks are not attributable to a further reduction of cytochrome b, which has a peak at 430 μm and not 426 μm; (b) the conversion of oxyhemoglobin to hemoglobin should occur on anaerobiosis, as in Fig. 1, and would not require addition of dithionite; (c) the differencespectrum of hemoglobin minus methemoglobin does not produce peaks with these maxima. Chance and Williams (7) have found a similar compound in intact mitochondria which is reduced by hydrosulfite and not by anaerobiosis, and conclude that it could be cytochrome b6, cytochrome (18), or unknown hemoproteins. It is possible that the digitonin treatment of the mitochondria denatures a part of the cytochromes. Hülsmann et al. (19) have demonstrated that the component in mitochondrial (18) responsible for its characteristic spectrum is derived from the cytochromes. The amount of this compound varies considerably among different digitonin preparations, and it can be concluded that it is probably not a member of the phosphorylating electron transport system in the preparation, but may be an adventitious, possibly denatured, hemoprotein.

The average concentration of the hydrosulfite reducible material, determined with the use of ΔE340–405 μm = 200 mM⁻¹ cm⁻¹ (7), was 2.6 μmoles per mg. of N, which is about equal to the concentration of cytochrome c in the digitonin preparation. This material can thus account for only a small portion of the noncytochrome iron present in these preparations.

Pyridine Nucleotide Transhydrogenase Activity of Enzyme Complex—Although DPNH is rapidly oxidized by the digitonin preparation (1, 2), it has been found that TPNH is only very slowly oxidized. However, the addition of a catalytic quantity of DPN⁺ appreciably accelerates the rate, suggesting the presence of pyridine nucleotide transhydrogenase, which Kaplan et al. (20) have shown to occur in rat liver mitochondria. Transhydrogenase activity of the mitochondrial subfragments was assayed in a system composed of the mitochondrial subfragments, Fe+++ cytochrome c, cyanide to inhibit reoxidation of reduced

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**Table I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mitochondrial subfragments</th>
<th>Normal liver mitochondria*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmole/mg. N</td>
<td>molar ratios</td>
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<tr>
<td>Cytochrome a₂</td>
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<td>1.0</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Cytochrome c (α₃)</td>
<td>2.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Flavoprotein</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.45</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Values for normal liver mitochondria from Chance and Williams (7).

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1 J. Stern, personal communication of unpublished method.
Ball and Cooper (21) have also demonstrated that transhydrogenase is tightly bound to particles from heart mitochondria and Kielley and Bronk (24) have found transhydrogenase activity in rat liver mitochondrial fragments prepared by sonic vibration. Thus the presence of the transhydrogenase in these integrated systems in a bound form supports the concept that the transhydrogenase has a specific function in the respiratory chain.

Intact liver mitochondria contain 30 to 40 moles of pyridine nucleotide per mole of cytochrome a, whereas the digitonin fragments contain less than one mole of rather specifically bound DPN, reactive with β-hydroxybutyric dehydrogenase, but not with malic dehydrogenase (17). This bound DPN has a much higher “turnover” than the total DPN pool in intact mitochondria (7) and, with the exception of DPN, the carriers are in approximately the same relative molar proportions. This was a rather unexpected observation, for it has been observed that other treatments which disrupt the morphology of mitochondria alter the relative proportions of the carriers (7–9). It may be concluded that the digitonin treatment fragments the mitochondrial membranes along relatively fragile lines of attachment between organized assemblies of phosphorylating respiratory enzymes (10, 22), without disturbing greatly the composition of the individual respiratory assemblies.

In the digitonin subfragments the rate-limiting step of the respiratory chain appears to be between cytochrome b and c. However, the relatively high degree of reduction of the cytochrome b which exists during the aerobic steady state may not be solely a reflection of its participation in electron transport, since it is possible that adventitious cytochrome b (i.e. “dislocated” from the chain) could be reduced rapidly by the electron transport system and remain in this state during respiration without undergoing rapid reoxidation. Chance (23) has presented evidence for reduction of “dislocated” cytochrome b in nonphotophorylating heart muscle preparations. It is probable that at least part of the cytochrome b in the digitonin fragments is in such a “dislocated” state, since the flavoprotein in the subfragments was not as fully reduced during the aerobic steady state oxidation as was cytochrome b. However, since approximately 55 per cent of the total cytochrome b in the digitonin subfragments becomes reduced when antimycin A is added to the system in the aerobic steady state, it may be concluded that a substantial fraction of the cytochrome b in these preparations is directly involved in electron transport.

The presence of pyridine nucleotide transhydrogenase in the mitochondrial subfragments supports the suggestion of Kaplan et al. (20) that the enzyme may link the TPN-reducing dehydrogenases with the DPN-linked system of phosphorylating electron transport. Since the digitonin subfragments contain the complete phosphorylating respiratory chain and only a few dehydrogenases, they can be considered as small subunits of the mitochondrial membrane containing only those enzymes which are closely associated with one another functionally. Ball and Cooper (21) have also demonstrated that transhydrogenase is tightly bound to particles from heart mitochondria and Kielley and Bronk (24) have found transhydrogenase activity in rat liver mitochondrial fragments prepared by sonic vibration. Thus the presence of the transhydrogenase in these integrated systems in a bound form supports the concept that the transhydrogenase has a specific function in the respiratory chain.

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specifically bound DPN on the phosphorylating respiratory assembly in the membrane. Kaplan et al. (25) have demonstrated that the TPN–DPN transhydrogenase of beef heart also transfers hydrogen from DPNH to DPN+. Experiments are in progress to determine whether rat liver mitochondria and the digitonin fragments prepared from them are also capable of carrying out this type of transhydrogenation.

SUMMARY

Difference spectra of phosphorylating subfragments of rat liver mitochondria, prepared by treatment with digitonin, were recorded under a variety of conditions. Cytochromes $a$, $a_3$, $b$, and $c$, and flavoprotein were identified as part of the respiratory chain in these particles. These carriers are completely reduced by $D(-)-\text{p-hydroxybutyrate}$ under anaerobic conditions. Bound diphosphopyridine nucleotide could not be directly measured in the difference spectrum, but was determined by an enzymatic assay. The relative concentrations of cytochromes $a$, $a_3$, $b$, and $c$, flavoprotein and diphosphopyridine nucleotide in the subfragments were found to be $1.0:1.0:0.7:1.8:3.0:0.3$, respectively. These proportions are very similar to those observed in intact rat liver mitochondria, with the exception of diphosphopyridine nucleotide, indicating that the phosphorylating fragments prepared with digitonin suffer very little damage to the respiratory assemblies during isolation. Some of the cytochrome $b$ present in the particles appears to be “dislocated” from the respiratory chain. The fragments also contain a pigment which is reducible by hydrosulfite but not by $\beta$-hydroxybutyrate; it does not appear to lie directly on the respiratory chain. The digitonin particles also contain a TPN–DPN pyridine nucleotide transhydrogenase which is inhibited by L-thyroxine. A special role of bound DPN and bound pyridine nucleotide transhydrogenase in phosphorylating electron transport is discussed.

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REFERENCES

The Respiratory Chain in Phosphorylating Subfragments of Mitochondria Prepared with Digitonin
Thomas M. Devlin