Studies on the Mechanism of Oxidative Phosphorylation

IX. EFFECT OF CYTOCHROME c ON ENERGY-LINKED PROCESSES*

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SUMMARY

1. Heavy bovine heart mitochondria were made deficient in cytochrome c by extraction with a solution of KCl. This preparation, containing about 15\% of the normal complement of cytochrome c, was used in studies on the effect of exogenous cytochrome c on several energy-linked processes.

2. The P:O ratio of the preparation was increased as the oxidation rate was increased with small progressive additions of cytochrome c. The effect may have been caused by the participation of cytochrome c in the formation of an intermediate of oxidative phosphorylation, or to the fact that the P:O ratios were lowered as the rate at which electrons traversed the chain was lowered.

3. In the cytochrome c-deficient particles, the energy-linked systems implementing calcium translocation and pyridine nucleotide transhydrogenation were found to be intact and fully functional when adenosine triphosphate was the source of energy. However, the systems required cytochrome c when oxidation of substrate was the source of energy.

4. The study points out the unlikelihood that cytochrome c is a component part of an intermediate that functions in the steps between the breakdown of ATP and the formation of the high energy intermediate utilized by energy-requiring processes. It may, however, be a component part of an intermediate that functions at a point between the electron transfer chain and the formation of the high energy intermediate utilized by energy-requiring processes.

Although the sequential steps of oxidative phosphorylation have not been elucidated, certain chemical sequences have been proposed. Basic to many of these schemes is the concept of a high energy bond formed between an electron transfer component and an unknown compound. The most common designation for this intermediate is C~I (1), where C is a component of the electron transfer chain and I is an inhibitor of electron transfer. Although the reactions comprising oxidative phosphorylation occur in a lipid-protein membrane which is water-insoluble, some of the proposed participants in the reaction can be extracted from the membrane. Bound pyridine nucleotides (2, 3), coenzyme Q (4), certain proteins (for example, the coupling factors) (5-8), and cytochrome c (9) can all be extracted from mitochondria, and their reintroduction into mitochondria or derivative particles restores some or all of the original membrane functions.

An evaluation of the role of cytochrome c as a component of a high energy intermediate can be made by first removing cytochrome c from the mitochondrion and then, upon the readdition of the cytochrome, by comparing the increments found in the restoration of energy-linked systems with those of electron transfer. The results of this line of investigation are presented in this communication.

MATERIALS AND METHODS

Preparation of Cytochrome c-deficient Mitochondria—Bovine heart mitochondria were prepared by the method of Crane, Glenn, and Green (10). Heavy mitochondria were isolated according to the method of Hatefi and Lester (11), except that the pH of the suspension was adjusted to 7.8 before centrifugation. Suspensions of heavy mitochondria, at a protein concentration of 40 mg per ml, were frozen for a period of 1 to several days at -20\° in a preserving medium which was 0.25 M sucrose, 0.01 M Tris-acetate, pH 7.5, 1 mM in ATP, 1 mM in MgCl₂, and 1 mM in succinate. When thawed, the suspension was adjusted to pH 7.8 and was centrifuged at 26,000 \times g for 10 min. The light layer of the pellet was discarded, and the heavy layer was suspended at a concentration of 20 mg per ml in a solution which was 0.015 M in KCl. The mitochondria were permitted to swell in the hypotonic medium for 10 min, and then were centrifuged at 105,000 \times g for 15 min. The colorless supernatant fluid and a small dark layer of unswollen mitochondria at the bottom of the tube were discarded. The swollen mitochondria were suspended at a protein concentration of 30 mg per ml in 0.15 M KCl. The mitochondria, left in the isotonic KCl for 10 min, were then centrifuged at 105,000 \times g for 15 min. The red supernatant fluid containing most of the extractable cytochrome c was decanted and the extraction with isotonic KCl was repeated twice more. After the final centrifugation a small fluffy layer of light mitochondria, a fraction low in phosphorylative capacity and representing the main contaminant in the otherwise homogeneous

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mitochondrial population, was removed. The mitochondria of the densely packed heavy layer, deficient in cytochrome c, were suspended at a protein concentration of 20 mg per ml in a solution which was 0.25 M in sucrose and 0.01 M in Tris-acetate buffer, pH 7.5. The extraction procedure was essentially the same as that described by Jacobs and Sanadi (9) for rat liver mitochondria.

The cytochrome c content of the extract was determined spectrophotometrically with a coefficient of 18.5 mm"1 cm"1 for the difference in absorbance at 550 nm between the oxidized and dithionite-reduced forms. In cases where the cytochrome c content of the extracts was too low for accurate determination, the cytochrome c was concentrated by adsorption to and elution from Amberlite CG-50 resin. The resin was processed according to the method of Hagihara et al. (12); the KCl extracts were passed through a column, 2 x 2 cm, of the resin. The cytochrome c which was adsorbed in a narrow zone at the top of the column was subsequently eluted quantitatively with a few milliliters of 10% ammonium sulfate, pH 8.0. Cytochrome c determinations were then made on these concentrated solutions.

**Phosphorylation Assays**—Phosphorylation assays involving oxygen uptake were performed either in the standard Warburg respirometer or in an oxygraph (Gilson Medical Electronics) equipped with a 2-ml cell.

Manometric experiments were performed in the manner described by Lenaz and Beyer (13), with the exception that 2 mg of bovine serum albumin were added to each Warburg flask. Assays for phosphorylation in the span, cytochrome c to oxygen, were carried out in the presence of 40 μmoles of ascorbate and either 150 μmoles of N,N',N',N'-tetramethyl-p-phenylenediamine or 3 μmoles of phenazine methosulfate. Antimycin A, 1 μg per mg of mitochondrial protein, was added. Cytochrome c was added, where indicated, at a level of 5 μg per mg of mitochondrial protein. The reactions were initiated by the addition of substrates from the side arm of the flask.

For the oxygraph assay, the reaction mixture contained, in a volume of 2 ml, 500 μmoles of sucrose, 50 μmoles of glucose, 10 μmoles of MgCl₂, 6 μmoles of ADP, 100 μg of hexokinase ( Worthington), 20 μmoles of potassium phosphate labeled with 32P (approximately 200 000 cpm per μmole), 4 μg of mitochondrial protein, and varying amounts of cytochrome c (Sigma type III). Bovine serum albumin, 1 mg per ml, was added to some assays. Substrate additions were (a) succinate, 20 μmoles; (b) pyruvate plus malate, 5 μmoles of each; or (c) ascorbate, 20 μmoles, plus TPD, 0.15 μmole in the presence of 1 μg of antimycin A per mg of mitochondrial protein. Phenazine methosulfate (3 μmoles) was also used instead of TPD, with the same results. In the absence of mitochondrial protein, the uptake of oxygen was negligible in the presence of phenazine methosulfate or TPD at the concentrations indicated.

After all of the oxygen in the cell was consumed, 0.2-ml samples of the reaction mix were assayed for organic phosphate by the method of Lindberg and Ernster (14). KIO₃ (3 μmoles) was added to each tube prior to separation of inorganic from organic phosphates. After removal of the upper phase (isobutyl alcohol-benzene, 1:1, v/v), the lower phase was extracted twice more with water-saturated isobutyl alcohol. A sample of the lower phase was plated on a nickel planchet, dried, and counted in a thin window gas flow counter (Nuclear-Chicago). The formation of organic phosphate was calculated from these data by comparison with the specific activity of the inorganic phosphate added to the reaction mixture. Incorporation of 32P into organic phosphate in the absence of substrate oxidation was quantified in a sample of the reaction mixture to which 2 μg of antimycin A per mg of protein were added in place of substrate.

Electron transfer, in that segment of the chain extending from pyruvate and malate to ferricyanide in the presence of cyanide, was assayed spectrophotometrically by modifications of the method of Estabrook (15). Phosphorylation was estimated by measurement of the incorporation of 32P into organic phosphate. The assay medium, in a total volume of 3 ml, contained glucose, 100 μmoles; sucrose, 750 μmoles; MgCl₂, 15 μmoles; potassium phosphate, 32P, buffer, pH 7.4, 10 μmoles; bovine serum albumin, 2 mg; ADP, 5 μmoles; hexokinase, 100 μg; mitochondrial protein, 1 mg; potassium ferricyanide, 1.0 or 1.5 μmoles; and KCN, 6 μmoles. The reaction was initiated by the addition of pyruvate plus malate (10 μmoles of each) after thermal equilibration for 3 min at 37°. The change in absorbance at 420 mμ was followed. The reaction was stopped by the addition of 0.1 ml of 50% perchloric acid after all of the ferricyanide was reduced (8 to 12 min). The phosphate incorporation values were corrected by subtracting the phosphate incorporation measured in a control without ferricyanide and without substrate.

**Oxygen Uptake and Oxidation**—The oxygraph recorder was calibrated with deionized water equilibrated with air at 30°; the oxygen contents of the reaction mixes were calculated by comparison with those of air-saturated water. Oxidation rates were calculated from data obtained with the oxygraph or by manometric measurements.

**Calcium Translocation Assay**—Calcium uptake was determined in a system similar to that described by Brierley, Murer, and Baehmann (16). Details of the experiments are given in the legends to Figs. 2 and 3.

**Pyridine Nucleotide Transhydrogenation**—Because the transhydrogenation reaction can be measured more simply and more quantitatively in submitochondrial particles, electron transfer particles were prepared from KCl-HBHM by sonic oscillation for 60 sec in the presence of the addends, 1 mM succinate, 1 mM ATP, 5 mM MgCl₂ and 10 mM MnCl₂ as described by Hansen and Smith (17).

Transhydrogenation was assayed by the method of Lee and Ernster (18) with certain modifications. The assay medium contained, in a volume of 3 ml, Tris-sulfate buffer, pH 7.5, 150 μmoles; MgCl₂, 18 μmoles; succrose, 750 μmoles; bovine serum albumin, 2 mg; DPNH, 0.5 μmole; and either succinate, 10 μmoles, or ATP, 10 μmoles. DPNH was regenerated by a method similar to that described by Danielson and Ernster (19) in a system consisting of 0.62 ml of ethanol, 300 μg of alcohol dehydrogenase, 4 μg of rotenone, and 0.5 μg of ETF₄₇ protein. The temperature of the reaction mixture was maintained at 37°. The reaction was started by the addition of 0.6 μmole of TPNH and was followed by measurement of absorbance at 340 μμ in a Beckman model DU spectrophotometer; readings were taken at intervals of 60 sec. The rate of transhydrogenation was calculated as millimicromoles of TPNH formed per min per mg of protein during the period in which the rate was linear.
Reduction of Endogenous DPN⁺ by Succinate—The reduction of endogenous DPN⁺ by succinate was carried out at room temperature in a system which contained, in a final volume of 3 ml: sucrose, 500 μmoles; triethanolamine-HCl buffer, pH 7.2, 40 μmoles; EDTA, 4 μmoles; arsenite, 3 μmoles; ATP, 5 μmoles; succinate, 15 μmoles; and mitochondrial protein, 21 mg. The medium contained, per mg of protein, either 0.5 μg of antimycin A or 1 μg of oligomycin. The medium was flushed with nitrogen before the reaction was started by the addition of mitochondrial protein. The mixture was then aerated for 30 sec and was incubated for an additional 3 min. The reaction was stopped by the addition of 0.6 ml of 3 N perchloric acid, nitrogen being bubbled through the medium to ensure mixing. The oxidized DPN⁺ in the acid extract was estimated enzymatically with alcohol dehydrogenase as described by Klingenberg (20). The amount of DPN⁺ reduced in the assay was calculated from the amounts of the oxidized forms found in the experimental and in the control (containing both antimycin and oligomycin) samples.

Protein was determined by the biuret method of Gornall, Bardawill, and David (21). ⁴⁴CaCl₂ was obtained from the General Electric Company.

RESULTS

Effect of Cytochrome c Depletion on Oxidation Rates

Heavy bovine heart mitochondria, swollen in hypotonic KCl, readily lost most of their cytochrome c when extracted with isotonic KCl. This result is similar to that reported by Jacobs and Sanadi (9) for rat liver mitochondria. The amount of cytochrome c extracted from the mitochondria was about 0.385 μmole per mg of protein (cf. Table I). Since mitochondria contain about 0.45 μmole of cytochrome c per mg of protein (22), the extraction removed about 85% of the cytochrome c. An independent estimate of the amount of cytochrome c removed from the mitochondria was obtained by comparison of the original and the residual oxidation rates. With pyruvate plus malate as substrate, the oxidation rates varied in different preparations between about 0.02 and 0.03 μatom of oxygen consumed per min per mg of protein, which is about 10 to 15% of the rate found for the initial mitochondrial preparations. These two estimates would indicate that about 15% of the native cytochrome c remained in the KCl-extracted mitochondria.

Effect of Cytochrome c Depletion on Oxidative Phosphorylation

With particles deficient in cytochrome c we were able to examine the effect of added cytochrome c on both oxidation and phosphorylation. Table II presents results of experiments in which cytochrome c was added to deficient mitochondria. Cytochrome c caused an increase in the rates of both oxidation and phosphorylation with pyruvate plus malate, with succinate, and with ascorbate plus TPD (in the presence of antimycin) as substrate. With each substrate the increase in the rate of phosphorylation exceeded that of oxidation, and consequently the P:O ratios increased. In experiments of this type, relationships were difficult to reproduce among experimental variables. If protein was maintained constant (Experiment I), the time of the experiment or the total amount of oxygen consumed differed in the presence and absence of cytochrome c. If the protein concentration was varied, as in Experiment II, time of the experiment and total oxygen consumption were more nearly the same in the presence and absence of cytochrome c. Nevertheless, when these parameters were varied, exogenous cytochrome c was found to increase the measured P:O ratios consistently. This effect of cytochrome c has not been reported by other workers who used either rat liver or rat heart mitochondria (9, 23).

In order to determine whether the effect of cytochrome c could be observed over a range of concentrations, graded amounts of cytochrome c were added to the deficient mitochondria and P:O ratios were measured with succinate as substrate (cf. Table III). Upon the addition of increasing amounts of cytochrome c, the increments in the restoration of phosphorylation exceeded those of electron transfer, and the P:O ratio was increased, finally by a factor of more than 3. The increment in P:O ratios occurred over a wide range of cytochrome c concentrations.

In an attempt to determine the site specificity of the cytochrome c effect, a number of determinations were made with...
uptake was measured as incorporation of $^{32}$P into organic compounds (see text). The substrate was succinate.

**Experiment I**

Average $P:O$ ratios with KCl-HBHM in presence and absence of organic compounds. The substrate was succinate.

| KCl-HBHM 1.5 | 1.5 | 2.5 | 140 | 104 | 1.58 |
| KCl-HBHM 2.5 | 2.5 | 2.5 | 104 | 104 | 1.45 |
| KCl-HBHM 3.0 | 3.0 | 3.0 | 104 | 104 | 1.45 |

**Experiment II**

The presence or absence of cytochrome $c$ did not affect the $P:O$ ratios when phosphorylation was measured during the transfer of electrons between DPNH and ferricyanide in a cyanide-blocked system (cf. Table V). The addition of cytochrome $c$, however, did not increase the rate of reduction of ferricyanide, a result in contrast to the effect shown by Estabrook (15). That electrons were passing through both phosphorylation sites, I and II, is implicit from the fact that the $P:O$ ratio measured in the presence and in the absence of cytochrome $c$ was 1.5.

While the results with succinate, with ascorbate plus TPD, and with pyruvate plus malate in the presence of ferricyanide and cyanide are consistent with the hypothesis that the effect of cytochrome $c$ is confined to Site III only, the results with pyruvate plus malate in the presence of oxygen as electron acceptor are not. Thus an average $P:O$ increase of 1.4 indicates that the effect of cytochrome $c$ is seen at more than one site.

The presence or absence of cytochrome $c$ greatly affects the oxidation and phosphorylation rates. Thus, if the oxidation rates are low, even a slow hydrolysis of ATP or of glucose 6-phosphate might result in an apparent decrease in phosphorylation efficiency. We have measured these hydrolytic activities under conditions nearly identical with those of the oxidative phosphorylation assay (cf. Table VI). The endogenous mitochondrial myokinase can form ATP from ADP at rates of up to 525 mmoles per min per mg of protein. The ATP so formed can be hydrolyzed by the mitochondrial ATPase, or the terminal phosphate can be incorporated into glucose 6-phosphate by the action of hexokinase. Hexokinase therefore competes with ATPase. It is generally accepted that hexokinase competes favorably with ATPase (24), and this has proven to be the case in our experiments. In addition to this competition, ADP is an inhibitor of mitochondrial ATPase (25). Thus the hydrolysis of ATP under the conditions of oxidative phosphorylation should be minimal. In the presence of hexokinase, the combined ATPase and glucose 6-phosphatase activities measured accounted for about 2.6 mmoles of phosphate hydrolyzed per min per mg of protein. This value is about one-tenth of the phosphorylation rate observed in the absence of cytochrome $c$.

**Table III**

Effect of added cytochrome $c$ on oxidation and phosphorylation in KCl-HBHM

<table>
<thead>
<tr>
<th>Cytochrome $c$ added</th>
<th>Rate of $O_2$ uptake</th>
<th>Rate of $P_i$ uptake</th>
<th>$P:O$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome $c$ added</td>
<td>$\mu$ mole/min/mg protein</td>
<td>$\mu$ mole/min/mg protein</td>
<td>$\mu$ mole/min/mg protein</td>
</tr>
<tr>
<td>Experiment I</td>
<td>0.026</td>
<td>0.008</td>
<td>0.28</td>
</tr>
<tr>
<td>0.12</td>
<td>0.040</td>
<td>0.013</td>
<td>0.32</td>
</tr>
<tr>
<td>0.24</td>
<td>0.040</td>
<td>0.008</td>
<td>0.20</td>
</tr>
<tr>
<td>0.35</td>
<td>0.065</td>
<td>0.025</td>
<td>0.46</td>
</tr>
<tr>
<td>0.48</td>
<td>0.085</td>
<td>0.025</td>
<td>0.45</td>
</tr>
<tr>
<td>0.58</td>
<td>0.083</td>
<td>0.040</td>
<td>0.50</td>
</tr>
<tr>
<td>0.83</td>
<td>0.087</td>
<td>0.059</td>
<td>0.69</td>
</tr>
<tr>
<td>1.70</td>
<td>0.106</td>
<td>0.081</td>
<td>0.81</td>
</tr>
<tr>
<td>4.20</td>
<td>0.168</td>
<td>0.160</td>
<td>1.01</td>
</tr>
<tr>
<td>8.40</td>
<td>0.149</td>
<td>0.156</td>
<td>1.05</td>
</tr>
<tr>
<td>Experiment II</td>
<td>0.032</td>
<td>0.013</td>
<td>0.41</td>
</tr>
<tr>
<td>0.17</td>
<td>0.054</td>
<td>0.025</td>
<td>0.51</td>
</tr>
<tr>
<td>0.33</td>
<td>0.093</td>
<td>0.072</td>
<td>0.77</td>
</tr>
<tr>
<td>0.67</td>
<td>0.150</td>
<td>0.127</td>
<td>0.84</td>
</tr>
<tr>
<td>1.00</td>
<td>0.172</td>
<td>0.173</td>
<td>1.01</td>
</tr>
<tr>
<td>1.67</td>
<td>0.214</td>
<td>0.196</td>
<td>0.91</td>
</tr>
<tr>
<td>3.33</td>
<td>0.170</td>
<td>0.301</td>
<td>1.88</td>
</tr>
</tbody>
</table>

- Oxygen uptake was measured in the oxygraph and phosphate uptake was measured as incorporation of $^{32}$P into organic compounds (see text). The substrate was succinate.
- Oxygen uptake was measured in the Warburg apparatus and phosphate uptake was measured as incorporation of $^{32}$P into organic compounds. The substrate was succinate.

**Table IV**

Average $P:O$ ratios with KCl-HBHM in presence and absence of cytochrome $c$ with different substrates

Oxygen consumption was measured manometrically and esterification of phosphate was measured colorimetrically, as described in the text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Without cytochrome $c$</th>
<th>With cytochrome $c$, 5 $\mu$g per mg of protein</th>
<th>$P:O$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome $c$</td>
<td>$\mu$ mole/min/mg protein</td>
<td>$\mu$ mole/min/mg protein</td>
<td>$\mu$ mole/min/mg protein</td>
</tr>
<tr>
<td>Pyruvate + malate</td>
<td>0.022</td>
<td>0.025</td>
<td>1.14</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.055</td>
<td>0.025</td>
<td>0.71</td>
</tr>
<tr>
<td>Ascorbate (TPD or phenazine methosulphate)</td>
<td>0.027</td>
<td>0.009</td>
<td>0.24</td>
</tr>
</tbody>
</table>

pyruvate plus malate (assay for Sites I, II, and III), succinate (Sites II and III), and ascorbate plus TPD (Site III) as substrates. With DPNH linked substrates, cytochrome $c$ increased the oxidation rate some 8.6-fold and the $P:O$ ratio by 1.26 (cf. Table IV). With succinate as substrate, the oxidation rate was increased 4-fold and the $P:O$ ratio was increased by 0.35. With ascorbate (plus TPD) as substrate, the oxidation rate was increased 4.5-fold and the $P:O$ ratio was increased by 0.39.

The presence or absence of cytochrome $c$ did not affect the $P:O$ ratios when phosphorylation was measured during the transfer of electrons between DPNH and ferricyanide in a cyanide-blocked system (cf. Table V). The addition of cytochrome $c$, however, did not increase the rate of reduction of ferricyanide, a result in contrast to the effect shown by Estabrook (15). That electrons were passing through both phosphorylation sites, I and II, is implicit from the fact that the $P:2e$ ratio measured in the presence and in the absence of cytochrome $c$ was 1.5.

While the results with succinate, with ascorbate plus TPD, and with pyruvate plus malate in the presence of ferricyanide and cyanide are consistent with the hypothesis that the effect of cytochrome $c$ is confined to Site III only, the results with pyruvate plus malate in the presence of oxygen as electron acceptor are not. Thus an average $P:O$ increase of 1.4 indicates that the effect of cytochrome $c$ is seen at more than one site.

The presence or absence of cytochrome $c$ greatly affects the oxidation and phosphorylation rates. Thus, if the oxidation rates are low, even a slow hydrolysis of ATP or of glucose 6-phosphate might result in an apparent decrease in phosphorylation efficiency. We have measured these hydrolytic activities under conditions nearly identical with those of the oxidative phosphorylation assay (cf. Table VI). The endogenous mitochondrial myokinase can form ATP from ADP at rates of up to 525 mmoles per min per mg of protein. The ATP so formed can be hydrolyzed by the mitochondrial ATPase, or the terminal phosphate can be incorporated into glucose 6-phosphate by the action of hexokinase. Hexokinase therefore competes with ATPase. It is generally accepted that hexokinase competes favorably with ATPase (24), and this has proven to be the case in our experiments. In addition to this competition, ADP is an inhibitor of mitochondrial ATPase (25). Thus the hydrolysis of ATP under the conditions of oxidative phosphorylation should be minimal. In the presence of hexokinase, the combined ATPase and glucose 6-phosphatase activities measured accounted for about 2.6 mmoles of phosphate hydrolyzed per min per mg of protein. This value is about one-tenth of the phosphorylation rate observed in the absence of cytochrome $c$. 

**Table V**

Effect of cytochrome $c$ deficiency on phosphorylation coupled to transfer of electrons between DPNH and ferricyanide

The assay was carried out as described in the text.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Peri-cyanide</th>
<th>Cytochrome $c$</th>
<th>Rate of $1e^-$ transfer $^a$</th>
<th>Rate of $P_i$ uptake $^a$</th>
<th>$P/2e^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBBM $^e$</td>
<td>1.5</td>
<td>154</td>
<td>125</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>HBBM $^e$</td>
<td>1.5</td>
<td>2.5</td>
<td>140</td>
<td>111</td>
<td>1.45</td>
</tr>
<tr>
<td>KCl-HBBM $^e$</td>
<td>1.5</td>
<td>2.5</td>
<td>94</td>
<td>67</td>
<td>1.45</td>
</tr>
<tr>
<td>KCl-HBBM</td>
<td>1.5</td>
<td>2.5</td>
<td>104</td>
<td>77</td>
<td>1.46</td>
</tr>
<tr>
<td>KCl HBBM</td>
<td>1.5</td>
<td>2.5</td>
<td>64</td>
<td>46</td>
<td>1.45</td>
</tr>
<tr>
<td>KCl HBBM</td>
<td>1.0</td>
<td>2.5</td>
<td>96</td>
<td>64</td>
<td>1.49</td>
</tr>
</tbody>
</table>

- The activity is expressed as millimicroequivalents of electrons transferred per min per mg of protein.
- The rate of $P_i$ uptake is expressed as millimicromoles of $P_i$ esterified per min per mg of protein.
- The preparation contained 1 mg of protein per 3 ml.
...transfer. It has been intimately connected with an increase in the rate of electron rates (cf. Table III, for example). We cannot rule out the possibility, by analogy, in a series of experiments in which the oxidation rates were varied either by the use of inhibitors of electron transfer or by the rate at which electrons were introduced into the chain.

Fig. 1 presents the results of an experiment in which the P:O ratios of KCl-extracted mitochondria were measured in the presence of an optimal level of cytochrome c but with small additions, respectively, of cyanide, sulfide, azide, or malonate. Cyanide and sulfide caused sharp changes in the oxidation rate, so that it became difficult to titrate these ratios over a wide range. The data indicated, nevertheless, that the P:O ratio remained fairly constant until the rate of oxidation was lowered to a value of about 40 mmoles of oxygen consumed per min per mg of protein. As rates below this point the P:O ratio declined rapidly. This effect was probably not the result of uncoupling, because a different effect was observed with azide, an agent which is known to be an uncoupler of oxidative phosphorylation (26, 27) as well as an inhibitor of oxidation. With azide, the drop in P:O ratio occurred over a wide range of inhibition, the rate of phosphorylation approaching zero when the oxidation rate was still relatively high. Malonate, in the presence of rotenone, had little effect on the P:O ratios over a wide range of inhibition of oxidation. A slight decrease was seen, however, when the rate of oxidation was lowered to about 20 mmoles of oxygen consumed per min per mg of protein. Malonate, a competitive inhibitor of succinic dehydrogenase, would probably not affect the rate at which electrons moved through the chain but would affect the rate at which succinate was oxidized. The fact that with malonate the P:O ratios did not fall off drastically even at low rates of oxidation indicates that the efficiency of phosphorylation depends more on the rate at which electrons traverse the chain than on the total time over which a given amount of substrate is oxidized.

In general, the effect of low rates of oxidation, when induced by inhibition, was to lower the P:O ratio. This effect seemed to occur only when the rate of oxidation was low (40 mmoles or less of oxygen consumed per min per mg of protein). We have frequently observed increases in P:O, when induced by the addition of cytochrome c, over a much wider range of oxidation rates (cf. Table III, for example). We cannot rule out the possibility, however, that this effect of cytochrome c may have been intimately connected with an increase in the rate of electron transfer.

**Table VI**

<table>
<thead>
<tr>
<th>Phosphatase activity</th>
<th>With hexokinase</th>
<th>Without hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>1.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Glucose 6-phosphatase</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Myokinase</td>
<td>525</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** The effect of the rate of oxidation on the P:O ratios of KCl-extracted mitochondria. The assay system with succinate as substrate was described under “Materials and Methods.” All assays were performed on the same preparation of particles. Cytochrome c was present at a level of 5.5 μg per mg of mitochondrial protein. The range over which the concentrations of inhibitor were varied was: azide, 0 to 3000 μM (○); malonate, 0 to 6000 μM (●) (in the presence of 1 μg of rotenone per mg of mitochondrial protein (△)); sulfide, 0 to 5 μM (□); cyanide, 0 to 170 μM (●).

Further experiments reported in this communication are concerned with the effect of cytochrome c on other energy-linked processes, in particular those driven both by ATP and by oxidation of substrate.

**Effect of Cytochrome c on Calcium Translocation**

ATP-supported—The cytochrome c-deficient mitochondria retained the capacity to translocate calcium ions at the rate of 180 mmoles per min per mg of protein when ATP was the energy source. The rate was not affected by the addition of cytochrome c (Fig. 2, Experiment A). The rate could be in-
Experimental conditions were similar to those described in the legend to Fig. 2. Four micrograms of oligomycin replaced 4 μg of ATP and calcium were added to the flasks to start the reaction. ATP and calcium were added to start the reaction. In Experiment C, the mitochondria were incubated with both ATP and succinate. Antimycin A was added after 5 min, the uptake of calcium being driven by ATP as the energy source. The initial rates of ion uptake were high but were no longer linear; in 2 min much less calcium was translocated than under conditions for A and B. Cytochrome c prevented this degeneration in rate when it was included in the medium during the initial incubation, but it had no effect when added subsequently to the addition of antimycin. In Experiment D, the mitochondria were incubated with succinate for 3 min before antimycin was added. ATP and calcium were added to start the reaction. Again, an impairment of the uptake process was observed, this impairment being partially alleviated by cytochrome c.

Translocation of calcium ions by KCl-extracted mitochondria, when driven by ATP, did not require added cytochrome c. Also, the initial incubation (prior to ion uptake) of the cytochrome c-deficient particles with succinate, or with succinate plus ATP, led to an impairment of the translocation process. When cytochrome c was present during this initial incubation period it partially prevented the impairment. Presumably, cytochrome c increased the rates of electron transfer and of the generation of the high energy intermediates which aid in the maintenance of the calcium uptake system.

Sucinate-supported—When calcium translocation was driven by the electron transfer process with succinate as the electron donor, a clear requirement for cytochrome c emerged. As shown in Fig. 3, Experiments A and B, the particles took up almost no calcium in the absence of added cytochrome c. The increases in the rates of calcium uptake and of oxygen uptake were about the same. We have not attempted to measure Ca:O ratios because of the nonlinearity of the reactions with time; also, the respiratory control values in the KCl-extracted mitochondria were too low to permit accurate measurement of the Ca:O ratio by the oxygen uptake stimulated by Ca++. The rates of calcium uptake were enhanced when the mitochondria were incubated with succinate for 4 min before calcium was added. In this case exogenous cytochrome c increased the rate of calcium uptake, and this effect was seen whether cytochrome c was added before or after the preincubation. In Experiment D, the mitochondria were incubated with both ATP and succinate. Oligomycin was added after 3 min and calcium at 4 min to start the reaction.

From these observations we cannot conclude whether cytochrome c has an effect on calcium translocation above and beyond its effect of increasing the rate of generation of a high energy intermediate because we have been unable to measure incisive Ca:O ratios, as we did P:O ratios for oxidative phosphorylation.

**Effect of Cytochrome c on Pyridine Nucleotide Transhydrogenation**

Transhydrogenation between pyridine nucleotides, another energy-linked reaction which can be driven either by ATP or by substrate (18, 19), cannot be measured in intact mitochondria.
but is readily measured in a derived submitochondrial particle. We have prepared ETPH from KCl-HBHM by sonic oscillation in the presence of Mg++, Mn++, ATP, and succinate (17), and have measured transhydrogenation in the derived particle. A further description of the effects of cytochrome c on energy-linked processes in submitochondrial particles is presented in the following article (28).

Table VII presents data on the effect of cytochrome c deficiency on transhydrogenation. In ETPH prepared for HBHM, transhydrogenation rates were equally rapid whether the reaction was supported by energy from ATP or from substrate. Cytochrome c affected neither system. When the ETPH were prepared from mitochondria deficient in cytochrome c, the transhydrogenation rates were low when supported by substrate but were similar to those of ETPH prepared from HBHM of normal cytochrome c content when supported by ATP. Addition of cytochrome c affected neither system.

From these data we have concluded that in ETPH the rate of transhydrogenation, like the rate of calcium translocation in mitochondria, was unaffected by cytochrome c deficiency when the source of energy was ATP; however, the rates of both reactions were affected by cytochrome c deficiency when the source of energy was the oxidation of substrate.

Effect of Cytochrome c on Reversed Electron Transfer

In KCl-extracted mitochondria, reduction of endogenous DPN+ by succinate was observed (cf. Table VIII). The reaction occurred in the presence, and also in the absence, of exogenously added cytochrome c. More DPNH was formed, however, in the presence of cytochrome c than in its absence when the source of energy was succinate oxidation. We have observed very little reduction of DPN+ by succinate in an antimycin-blocked system when ATP was the energy source.

Table VII

<table>
<thead>
<tr>
<th>Particle and additiona</th>
<th>Reduction of TPN+b by energy source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate</td>
</tr>
<tr>
<td></td>
<td>mmoles TPN reduced/min/mg protein</td>
</tr>
<tr>
<td>ETPH(Mg, Mn)b</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>40</td>
</tr>
<tr>
<td>Energy source</td>
<td>156</td>
</tr>
<tr>
<td>Energy source and cytochrome c++</td>
<td>145</td>
</tr>
<tr>
<td>ETPH from KCl-HBHMc</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>17</td>
</tr>
<tr>
<td>Energy source</td>
<td>44</td>
</tr>
<tr>
<td>Energy source and cytochrome c++</td>
<td>42</td>
</tr>
</tbody>
</table>

a When the reaction was driven by ATP, 2 μg of antimycin A per mg of mitochondrial protein were used. When the reaction was driven by succinate, 5 μg of oligomycin per mg of protein were used.

b Prepared by the method of Hansen and Smith (17).

c The concentration of cytochrome c was 4.2 μg per mg of mitochondrial protein.

d ETPH (Mg, Mn) was prepared by the method of Hansen and Smith (17) with KCl-HBHM as starting material.
competes favorably with ATPase for ATP formed during phosphorylation (24).

Since a breakdown of the phosphorylated end product does not seem to be the cause of low P:O ratios in the cytochrome c-deficient particles, the low P:O ratios must result from a lesion at the level of high energy intermediates. One possibility is that formation of high energy intermediates at Site III may be dependent upon the presence of a sufficient level of cytochrome c to fulfill a role both in electron transfer and in the formation of intermediates.

A second possibility is that the rate of electron transfer or the state of reduction of the electron transfer chain determines, to some extent, the efficiency of formation of the intermediate. We have examined this possibility and have found, by using inhibitors, that the P:O ratios are lowered when the rates of oxidation are low. This phenomenon occurs when the rate of consumption of oxygen falls below about 40 nmoles/min per mg of protein. The effect of cytochrome c in increasing P:O ratios was observed frequently over a wide range of oxidation rates. The possibility that the effect of cytochrome c in increasing the P:O ratios is due to its effect on the rate of electron transfer, however, cannot be ruled out as the explanation of the phenomenon.

Hydrolysis of high energy intermediates could be the cause of the association between low P:O ratios and low rates of electron transfer. Thus a constant rate of hydrolysis of intermediates at all rates of electron transfer would result in a greater proportion of high energy intermediates hydrolyzed at low rates of electron transfer. Such an effect should occur irrespective of the cause of lowered rates of electron transfer. In experiments in which oxidation rates were varied with inhibitors it was noted that malonate, which limits the rate at which electrons enter the chain, did not lower the P:O ratios appreciably at low rates of oxidation. From these data it can be deduced that low P:O ratios associated with low oxidation rates probably do not result because a greater proportion of high energy intermediates are hydrolyzed when the rates of oxidation are low.

Increased P:O ratios upon addition of cytochrome c to deficient particles could result if only a small population of the deficient particles retained the residual cytochrome c which enabled them to carry on basal oxidations without concomitant phosphorylation. The addition of cytochrome c to the whole population would restore oxidation and concomitant phosphorylation at equal rates, but the measured P:O ratio would increase because of the basal uncoupled oxidation. A study relevant to this question is concerned with the homogeneity of the population of mitochondria. We have examined the effects of cytochrome c on oxidation and phosphorylation in the most disparate populations of mitochondria (light and heavy) and have found that the P:O ratio is increased by cytochrome c in both types of mitochondria (23). This result would indicate that even if we were not dealing with a pure population of heavy mitochondria, the P:O ratio would still be increased in the whole population. The residual oxidation rate after KCl extraction was decreased some 75 to 100% with antimycin A; therefore, it is mitochondrial in origin. The simple explanation that increases in P:O arise from a dilution of nonphosphorylating oxidation then appears improbable.

From the studies reported in this communication it can be concluded that the systems which implement the energy-linked processes, i.e. phosphorylation, calcium translocation, and pyridine nucleotide transhydrogenation, require cytochrome c for optimal efficiency when supported by the oxidation of substrate. The same systems in cytochrome c-deficient particles are intact and fully functional when the source of energy is the hydrolysis of ATP, since the interaction of ATP with these energy-linked systems does not appear to require the addition of exogenous cytochrome c. It is conceivable, however, that the first and second phosphorylation sites may support energy-linked processes driven by ATP without the participation of cytochrome c at the third site. The dependence of high energy intermediate formation on cytochrome c is seen when the intermediate is formed by electron transfer, but not when it is formed from the hydrolysis of ATP. The participation of cytochrome c in the formation of the high energy intermediate would thus appear to be limited to an early stage in the sequence of reactions which collectively conserve the energy released upon substrate oxidation.

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David H. MacLennan, Giorgio Lenaz and Ludmilla Szarkowska


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