The Influence of Deuterium Oxide and Organic Solvents on the Interaction of Respiratory Chain Components*

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SUMMARY

1. The influence of D2O and organic solvents on the rate of oxidation of added substrates by heart muscle particles and tightly coupled rat liver mitochondria has been investigated. Solvents examined include glycerol, sucrose, dimethyl sulfoxide, dimethyl formamide, and ethylene glycol. Each of these solvents was found to induce a marked inhibition of respiratory chain activity.

2. The relative constancy in the steady state reduction level of the respiratory carriers at each increment of solvent concentration prevents the assignment of a single specific site of respiratory inhibition, and favors the hypothesis that the solvent effects are due to a "nonspecific" inhibition which operates in a similar manner at several sites in the respiratory chain.

3. No correlation was found between the inhibition of respiratory activity by solvents and the viscosity of the solvent reaction mixtures.

4. The organic solvent inhibition of the DPNH oxidase activity of heart muscle particles was a linear function of the water content of the solvent reaction mixtures in which the particles were suspended, and independent of the concentration, tonicity, or chemical nature of the solvent used.

5. It is suggested that several protonolytic reactions may be operative during the transfer of reducing equivalents from substrate to oxygen by way of the respiratory chain. The respiratory chain components mediating these reactions are in some manner accessible to, and markedly influenced by, the aqueous phase surrounding the particles, and therefore do not function entirely in a lipid nonaqueous milieu.

There have been many studies and much speculation on the structural and chemical organization of the respiratory chain of mitochondria since the original observations (1, 2) that cellular oxidase activities are firmly bound to particulate elements. Keilin (3), Keilin and Hartree (4), and Keilin and Slater (5), in their original studies on the function of cytochromes recognized the need for what they termed "spatial organization." Recently, the application of the electron microscope to the study of mitochondrial structure has led to important observations. First, the frequency of cristae in the mitochondria isolated from a variety of tissues is roughly proportional to the respiratory enzyme content (6). Second, inner membrane subunits of mitochondria have been described (7-12) which may be related to respiratory chain components or to the coupling factors (13, 14) of respiratory chain phosphorylation. Presently, one can only say that cytochromes and associated dehydrogenases appear to be associated with the lipid membranes of mitochondria and that the manner in which they are organized is unknown.

The present study was undertaken to examine in detail the behavior of the cytochrome system in D2O and organic solvent-water reaction mixtures in order to gain some insight into the relationship of respiratory chain components to their environment. The results suggest that water or protons play a major role in the interaction of the cytochromes during oxidative metabolism.

METHODS

Two types of beef heart muscle preparations were used. One type, designated HMP (heart muscle particles), was prepared by a modification of the method of Keilin and Hartree (15). The second type was the ETP1 preparation of Crane, Glenn, and Green (16). Full details of both the preparations are described elsewhere (17). Rat liver mitochondria were prepared in 0.25 M sucrose by the method of Schneider (18). Cytochrome oxidase was prepared from beef heart muscle by the method of Yonetani (19).

Oxygen consumption was measured either manometrically in Barcroft differential manometers equilibrated at 31° with 3.3 ml reaction medium volume, or polarographically by the oxygen electrode technique (20) with a Clark oxygen electrode. Changes in optical density were recorded with the spectrophotometric methods developed by Yang (21), Yang and Legallais (22), and Chance (23). In some experiments, kinetic measurements of cytochrome oxidation and reduction were made in an Aminco-Chance dual wave length spectrophotometer (American Instrument Company, Maryland). Fluorometric measurements of mitochondrial pyridine nucleotide were made with a modified Eppendorf photometer adapted for continuous recording (24). The viscosity of the solvent-buffer mixtures was measured in an Ostwald viscometer (25).
DPNH and ADP were purchased from Sigma. Rotenone was the product of K and K Laboratories. The solvents used were of the highest purity available commercially, and were used without further purification. They were obtained from the following sources: deuterium oxide (heavy water, >99.5%) was purchased from the General Dynamics Corporation, Liquid Carbonic Division, San Carlos, California; ethylene glycol, dimethyl formamide, dimethyl sulfoxide, polyethylene glycol 400, and N-methyl acetamide were obtained from Fisher; glycerol, sucrose, and potassium chloride from Baker; 2-chloroethanol from Eastman Organic Chemicals; and p-dioxane from the Matheson Company, Inc.

Protein concentration was determined by the biuret method (26) with bovine serum albumin as standard.

RESULTS

Studies with D₂O

Inhibition of Succinate and DPNH Oxidation by D₂O—Manometric or polarographic measurements of oxygen utilization with heart muscle preparations showed, in agreement with the results of Laser and Slater (27), that the rate of succinate oxidation was reduced by about 45% when reactions were carried out in D₂O rather than H₂O. The degree of inhibition was related to the concentration of D₂O in the manner shown in Table I. The polarographic studies showed that the inhibition by D₂O was established rapidly and was not dependent on the time of exposure of the particles to D₂O. Measurements of oxygen uptake with DPNH as substrate showed a degree of inhibition by D₂O which was comparable to that observed with succinate. The latter results suggested that the inhibition of oxygen uptake was exerted in a region of the respiratory chain common both to the succinate and to the DPNH oxidase systems, and that the extent of inhibition was independent of the rate of electron flux through the respiratory chain.

An attempt was made to determine the site of inhibition by D₂O according to the general principles of the "crossover theo

Table I

<table>
<thead>
<tr>
<th>Concentration of D₂O</th>
<th>Succinate oxidation</th>
<th>DPNH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of O₂ uptake</td>
<td>Inhibition of D₂O</td>
</tr>
<tr>
<td>%, %/s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>204</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>170</td>
<td>17</td>
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<tr>
<td>50</td>
<td>147</td>
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<tr>
<td>75</td>
<td>136</td>
<td>33</td>
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<tr>
<td>95</td>
<td>124</td>
<td>39</td>
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</tbody>
</table>

* Microliters of O₂ consumed per mg of protein per hour.

Inhibition of Cytochrome c Oxidation—Studies on the influence of D₂O on the activity of cytochrome c oxidase were carried out with the purified preparation of cytochrome oxidase (cytochromes a + a₃) described by Yonetani (19). Both oxygen uptake measurements and spectrophotometric determinations of steady state cytochrome kinetics were made on a system composed of neutralized sodium ascorbate, heart muscle cytochrome c, and purified cytochrome oxidase.

The differences observed when the reaction was carried out in a medium in which H₂O was replaced by D₂O are illustrated by the oxygen electrode tracings presented in Fig. 2. The rate of oxygen uptake was inhibited by about 50% when the activity of the system was measured in D₂O (solid curve) compared to H₂O (dashed curve). A series of experiments with varying ratios of D₂O and H₂O showed that a progressive increase occurred in the degree of inhibition of oxygen uptake as the D₂O
content increased, an effect comparable to that seen previously (Table I) with the DPNH oxidase and succinate oxidase activities of heart muscle particles. In other experiments, the spectrophotometric determination of cytochrome c oxidase activity with reduced cytochrome c as substrate revealed an inhibition of activity by D2O comparable to that observed during the polarographic assay.

Steady State of Cytochromes a, a3, and c—A number of spectrophotometric experiments were carried out with the purified cytochrome oxidase preparation to see whether there were any differences in the extent of steady state reduction of the a and a3 hemoproteins in the D2O-inhibited system. Two series of experiments were performed, which used two different initial concentrations of cytochrome c in the reaction mixture. These conditions provided a 5-fold difference in the rate of oxygen uptake. As summarized in Table II, the extent of steady state reduction of the cytochrome components was not significantly altered in the presence of D2O, even though the rate of electron transport was inhibited (cf. Fig. 2).

Alternative Acceptors—The rate of reduction of exogenous cytochrome c, potassium ferricyanide, or 2,6-dichlorophenol by either DPNH or succinate was inhibited less by D2O than when oxygen served as the terminal acceptor of reducing equivalents (Table III). In general, the inhibition by D2O of the rate of reduction of the alternative electron acceptors by DPNH or succinate was about one-half of that observed for the over-all oxidation rate of DPNH or succinate by oxygen. The large differences in the rates of reduction of the acceptors used, and the finding that the rate of reduction of ferricyanide was the only rate comparable to the reaction rate with oxygen, have been discussed by Massey and Singer (29).

Affinity for Succinate—Thorn (30) showed that the affinity of the succinate oxidase system of heart muscle preparations is about 1.5 times lower if tetradecutosuccinate is used as substrate in place of succinate. The Km for sodium succinate was reported to be 1.2 mM, and that for tetradeuterosuccinate, 2.2 mM. To determine whether a proton exchange reaction had occurred in the present experiments when succinate was added to ETP suspended in a 99% D2O solution, the influence of succinate concentration on the initial rate of oxygen uptake was measured polarographically with either H2O or D2O in the reaction mixture. The data obtained in these experiments is presented in Fig. 3, in the form of Lineweaver-Burk plots. It may be seen that, despite the reduction of the Vmax value in D2O by about 50%, the affinity for succinate in D2O and H2O showed no significant difference (Km = 0.7 mM). Thus it may be concluded that D2O was not interfering with the primary reaction between succinate and succinic dehydrogenase, and that the observed inhibition of succinate oxidation was confined to the subsequent reactions mediating the transfer of reducing equivalents to oxygen or to alternative electron acceptors.

### Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Wave length (nm)</th>
<th>Reduction in the steady state (mM)</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
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<tr>
<td>Cytochrome a3</td>
<td>445</td>
<td>26</td>
</tr>
<tr>
<td>Cytochrome a</td>
<td>600</td>
<td>53</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>550</td>
<td>27</td>
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</table>

### Table III

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acceptor</th>
<th>Substrate</th>
<th>Enzyme concentration (mM)</th>
<th>Rate of reduction (μmol/sec/mg protein)</th>
<th>Inhibition by D2O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome c (10 μM)</td>
<td>DPNH</td>
<td>0.7</td>
<td>0.21</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>Potassium ferricyanide (0.4 mM)</td>
<td>Succinate</td>
<td>0.34</td>
<td>0.14</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>2,6-Dichlorophenol (20 mM)</td>
<td>DPNH</td>
<td>0.34</td>
<td>0.032</td>
<td>27</td>
</tr>
</tbody>
</table>

Fig. 2. Influence of D2O on the polarographic assay of oxygen utilization by cytochrome oxidase. The reaction vessel contained 0.43 μM cytochromes a and a3 and 75 μM cytochrome c suspended in 0.1 M phosphate buffer (pH 7.1), and 3 mM KCl, and 1% Emasol. Final volume, 4.0 ml. The reaction was initiated by the addition of sodium ascorbate. Temperature, 25°C.
Effect of D₂O on Oxygen Uptake during Oxidative Phosphorylation—Oxidative phosphorylation was assayed by the oxygen electrode technique (32) in isotonic media prepared by dissolving the salts in either D₂O or H₂O. In Fig. 4, two of the electrode traces obtained during the oxidation of glutamate by tightly coupled rat liver mitochondria are presented. The rate of oxygen uptake in both the "active" (State 3) and the "resting" (State 4) condition was inhibited about 50% by D₂O, a value similar to that obtained with nonphosphorylating heart muscle preparations (cf. above). The calculation of P:O ratios from the traces, by the method of Chance and Williams (32), revealed less than a 10% difference in the two experiments (2.9 in H₂O and 3.1 in D₂O with glutamate as substrate). Although the increase in P:O ratio may not be significant, it is of interest that a slightly higher value for the P:O ratio was always obtained in D₂O, when either succinate, glutamate, or β-hydroxybutyrate was used as substrate.

Fig. 3. Lineweaver-Burk plots of data from experiments in which the influence of succinate concentration on the initial rate of oxygen uptake by heart muscle particles (HMP) was assayed polarographically. The 4-ml reaction mixture contained 14 mg of protein in 0.1 M potassium phosphate buffer, pH 7.1, and 5 mM KCl, prepared in either D₂O or H₂O, and varying concentrations of succinate. Temperature, 25°.

Fig. 4. Influence of D₂O on the rate of oxygen uptake during oxidative phosphorylation. Rat liver mitochondria (17 mg of protein) were suspended in isotonic buffers prepared in either D₂O or H₂O and containing 80 mM KCl; 5 mM MgCl₂; and 10 mM potassium phosphate, pH 7.4. The subsequent additions of glutamate and ADP induced the characteristic increases in oxygen uptake. The dashed curve represents the tracing obtained with the sample suspended in H₂O, while the solid curve shows the tracing when D₂O was used in the medium. Temperature, 20°.

Fig. 5. Influence of D₂O on the steady state reduction of intramitochondrial pyridine nucleotide. The fluorescence of the reduced pyridine nucleotides of mitochondria was recorded with a modified Eppendorf photometer. Rat liver mitochondria (6 mg of protein) were diluted in 1.9 ml of H₂O or D₂O buffers similar to those used in the experiments presented in Fig. 4. The characteristic transitions of the steady states of pyridine nucleotide from State 1 to 5, as described by Chance and Williams (28), were observed as changes in the magnitude of the fluorescence of the sample. Curve A represents an experiment carried out in H₂O buffer, while Curve B was obtained with D₂O buffer.

Fig. 6. Influence of Glycerol—Chance and Spencer (33) have reported

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Fig. 6. Influence of glycerol on the cytochrome reduction-reoxidation cycle. Reaction mixtures contained 0.1 M potassium phosphate buffer (pH 7.2), 6 mg of ETP protein, and the concentration of glycerol indicated above the individual tracings. The final volume was 3 ml. The reaction was initiated by the addition of 0.18 mM DPNH, and the cytochrome kinetics were determined by recording the change in absorbance in a dual wave length spectrophotometer set at 552 – 540 nm. Temperature, 22°C.

% Inhibition/ % Molarity

605–625 nm

551–540 nm

% Reduction in Steady State

605–625 nm

551–540 nm

Glycerol Concentration Molar

Fig. 7. Influence of glycerol concentration on DPNH oxidase activity and the steady state reduction of endogenous cytochrome components. The experimental conditions were the same as those specified in the legend to Fig. 6. The kinetics of cytochrome reduction were recorded in a dual wave length spectrophotometer set at 551 – 540 nm (cytochromes c + cl) or 605 – 625 nm (cytochrome a). The percentage inhibition of DPNH oxidase by glycerol was estimated by assuming that the activity at each glycerol concentration tested was inversely proportional to the area under the spectrophotometric curve. Two plots of percentage inhibition versus molarity are shown, which represent the degree of inhibition calculated from the spectrophotometric traces obtained at the two pairs of wave length settings used. The percentage steady state reduction at each glycerol concentration tested was determined by adding sufficient DPNH to exhaust the oxygen remaining after the reduction-reoxidation response to the first addition of DPNH had been completed. The spectrophotometric trace, obtained upon the second DPNH addition, showed the transition between the fully oxidized condition of the cytochrome components to the steady state reduction level, followed by an abrupt transition to the fully reduced state when the oxygen became exhausted. The percentage steady state reduction of cytochrome was calculated from the tracing by expressing the absorbance change in the transition from the fully oxidized state to the steady state level as a percentage of the over-all change between the fully oxidized and the fully reduced condition.

on the inhibition of succinate and DPNH oxidation by 50% (v/v) glycerol solutions in the absence of any apparent change in the steady state of the cytochrome components. The influence of glycerol concentration on the succinate oxidase activity of the Keilin and Hartree heart muscle preparation observed under our conditions indicated that an inhibition of 50% was obtained with a glycerol concentration of about 3 m (25% v/v).

In further studies on the influence of glycerol and other solvents on the respiratory chain we have preferred to use the electron transfer particle (ETP) preparation of Crane et al. (16), which contains an extremely active DPNH oxidase system (Q0 value, about 1000 μl of oxygen consumed per mg of biuret protein per hour). The very high affinity shown by this preparation towards DPNH enabled a precise cyclic response of cytochrome reduction and reoxidation to be recorded when an aliquot of DPNH solution was added to the particle suspension. Separate experiments with the oxygen electrode showed that the reoxidation of cytochrome was synchronized with the cessation of oxygen consumption after the added DPNH had been oxidized.

The spectrophotometric traces, depicting a single cytochrome (c + cl) reduction-reoxidation cycle recorded when equal samples of a stock ETP suspension were diluted into various phosphate buffer-glycerol solutions and treated with a fixed concentration of DPNH, are reproduced in Fig. 6. The traces obtained in the separate experiments have been superimposed in order to illustrate more clearly the influence of glycerol concentration on the cytochrome response. In each experiment, the addition of DPNH caused an upward deflection of the spectrophotometric trace, indicating an increase in absorbance due to the reduction of endogenous cytochrome c and cl. The extent of the initial deflection of the trace was largely independent of the glycerol concentration, whereas the duration of the steady state reduction increased with increasing glycerol concentration. The time taken for the signal to return to the initial level was indicative of the time required to oxidize the DPNH added (as confirmed independently by separate polarographic measurements). A convenient estimate of the degree of inhibition of DPNH oxidase by glycerol was obtained by assuming that the DPNH oxidase activity in each experiment was inversely proportional to the area under the spectrophotometric curve. This method of calculation was used to obtain the data presented in Fig. 7, which shows the influence of glycerol concentration on DPNH oxidase activity and the percentage steady state reduction of endogenous cytochromes present in the ETP preparation. It
was found that a linear relationship existed between glycerol concentration and the percentage inhibition of DPNH oxidase activity up to about 5 mM glycerol or 70% inhibition, and that no significant changes were observed in the per cent steady state reduction of cytochromes $c + c_1$, or cytochrome $a$, as the glycerol concentration was increased progressively to the 5 mM level. The glycerol concentration required for 50% inhibition of DPNH oxidase was between 2.7 and 3.2 mM, a value similar to that required for a comparable effect on succinate oxidation in Keilin-Hartree heart muscle particles (see above).

It seemed possible that the failure to observe changes in cytochromes $c + c_1$ or cytochrome $a$ steady state reduction in the glycerol-treated particles was due to a balanced inhibitory influence at both the oxidase and the dehydrogenase regions of the DPNH oxidase system. The latter hypothesis was excluded by experiments in which the steady state reduction of cytochromes $c + c_1$ was modified towards a more oxidized level by treatment of the preparation with rotenone (34, 35), or towards a more reduced level by treatment with azide (36). The concentrations of rotenone and azide used inhibited oxygen uptake in the absence of glycerol by about 50%, and induced changes in the steady state reduction of cytochromes $c + c_1$ that would be expected from their specific inhibitory action (Fig. 8). The influence of glycerol on the DPNH oxidase system, either in rotenone-treated or in azide-treated ETP, was similar to that previously observed with untreated ETP. Despite the pronounced change in steady state reduction induced by rotenone in the absence of glycerol, no further significant change in steady state reduction occurred when measurements were made in solutions also containing glycerol (Fig. 8). When azide-treated ETP was used, the relationship between glycerol concentration and inhibition of activity was similar but not identical with that observed in the absence of azide, although the relationship was still a linear one. A slight change in the steady state occurred when the activity of azide-treated ETP was measured in the presence of glycerol. It would seem that glycerol does have a small influence on the action of azide on the respiratory chain. Nevertheless, the data presented in Fig. 8 give strong support to the idea that the inhibition of DPNH oxidase activity by glycerol is due to a "nonspecific" inhibition of the respiratory chain, and that this effect can operate under a wide range of steady state conditions.

**Inhibition by Sucrose, Ethylene Glycol, Dimethyl Sulfoxide, and Dimethyl Formamide**—The inhibition of the succinate oxidase system of the Keilin and Hartree heart muscle preparation by hypertonic sucrose was reported by Slater and Cleland (37). They concluded that sucrose inhibited the system by impairing the accessibility of endogenous cytochrome $c$ to the other components of the system, and classified the influence of sucrose as that of a nonspecific physical inhibitor. We found that the influence of sucrose on DPNH oxidase was similar to that previously observed with glycerol, since up to 70% inhibition of the over-all activity by sucrose was not accompanied by any significant change in the steady state reduction of cytochrome $c + c_1$. A sucrose concentration of about 1.2 M was required for 50% inhibition of DPNH oxidation.

Among the other solvents examined for their influence on ETP, three were found to act as nonspecific inhibitors, as judged by their ability to inhibit strongly the over-all DPNH oxidase activity of the preparations without significantly influencing the steady state reduction of cytochrome $c + c_1$. These solvents, with the concentration of solvent required to produce 50% in-
Inhibition of DPNH oxidase, were as follows: ethylene glycol (4.7 M), dimethyl sulfoxide (1.7 M), and dimethyl formamide (3.6 M). Of the five solvents which acted as nonspecific inhibitors, the most effective on a concentration basis was sucrose and the least effective was ethylene glycol. The relationship between the molarity of solvent and percentage inhibition of DPNH oxidase activity is illustrated in Fig. 9. It is evident that the nonspecific inhibitory effects observed were not directly related to the molarity of the solvent. Neither were they related to the toxicity of the solvent, since for example equal concentrations of sucrose or glycerol solutions, which have an equal toxicity, had very different effects on the oxidase activity.

Effect of Solvent Viscosity In the past, the nonspecific inhibition of the respiratory chain by glycerol has been attributed to an increased viscosity of the phase in which the respiratory carriers interact (38). Accordingly we have measured the viscosity of the solvent mixtures used in our experiments and replotted the data for each solvent to show the relationship between the viscosity and the degree of inhibition of DPNH oxidase activity (Fig. 10). It is apparent that the plots obtained with the various solvents used does not fall on the same curve, and it may therefore be concluded that the viscosity of the solvent-phosphate buffer mixtures was not the common factor responsible for their nonspecific inhibitory effects.

Influence of Solvent Water Content The nature of the influence of nonspecific inhibitors on the respiratory chain became apparent when the percentage of DPNH oxidase inhibition was plotted against the water content of the various solvent mixtures used. The data obtained for each solvent were found to show a remarkably similar relationship when expressed in this form (Fig. 11). Thus it is concluded that the nonspecific inhibitory effect of the various solvents was due to a reduction in the water content of the reaction mixtures and was independent of the chemical nature of the organic compound used. In agreement with this hypothesis it was found that the activity of ETP samples, suspended in strongly inhibitory concentrations of the solvents, was fully restored upon separation of the particles by centrifugation and rewashing with 0.25 M sucrose. The latter experiment showed that the action of the inhibitors was reversible, and that the solvents were not acting by irreversibly extracting or modifying a constituent of the enzyme preparation. The latter finding is consistent with the observation that respiratory chain preparations are largely insensitive to dehydration treatments. We have found that, when ETP preparations were lyophilized to a light fawn powder and then rehydrated with 0.25 M sucrose, all the succinate oxidase and at least one-half of the DPNH oxidase activity of the untreated preparation was retained in the lyophilized rehydrated preparation. These experiments recall the similar behavior of freeze-dried heart muscle preparations described by Keilin and Hartree (39).

Influence of Other Solvents—During this study, the influence of several other solvents on the DPNH oxidase system was
Solvents which induce specific inhibition of respiratory chain

The 3-ml reaction mixtures contained 0.1 m potassium phosphate buffer (pH 7.2), 5.5 mg of ETP protein, and varying concentrations of the solvent specified. DPNH oxidation and steady state cytochrome c + c1 reduction were assayed in a dual wave length spectrophotometer set at 551 – 540 mp. Different ETP preparations were used in Experiment 1 and Experiment 2. Temperature, 22°.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration required for 30% inhibition of DPNH oxidase</th>
<th>Steady state reduction of cytochromes c + c1</th>
<th>No solvent present</th>
<th>At 50% inhibition of DPNH oxidase by solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Chloroethanol</td>
<td>0.32 M</td>
<td>32</td>
<td>10</td>
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</tr>
<tr>
<td>Polyethylene glycol 400</td>
<td>8.5% v/v</td>
<td>31</td>
<td>17</td>
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<tr>
<td>KC1</td>
<td>0.3 M</td>
<td>34</td>
<td>48</td>
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</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>p-Dioxane</td>
<td>0.15 M</td>
<td>39</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>N-Methyl acetamide</td>
<td>1.3 M</td>
<td>40</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

investigated. These solvents were found to induce specific inhibitions of the respiratory chain, i.e. the inhibition of DPNH oxidase which they induced was associated with a significant change in the steady state reduction level of the cytochrome c + c1 components during DPNH oxidation. The data obtained with these solvents is listed in Table IV.

DISCUSSION

The present study was undertaken to gain information about the interaction of the cytochromes in the mitochondrial lipid membrane. The results described illustrate the nonspecific inhibitory effect of a number of solvents. It is remarkable that as much as 70% inhibition of the over-all activity of substrate oxidations through the respiratory chain can be achieved without causing any significant alteration in the steady state reduction level of electron transfer components. These results raise a number of questions about previous considerations of cytochrome organization.

1. The concept (40) of cytochromes insulated in a nonaqueous milieu, inaccessible to the solvent, appears untenable with the present results.

2. The hypothesis (33) that solvent viscosity modifies the molecular collisions of cytochrome molecules is not consistent with the data presented here.

3. The only common property of the solvents examined appears to be the reduction of the water content of the reaction media.

The nature of the influence of water on respiratory chain reactions can at present be discussed only in general terms. The following hypotheses appear to be consistent with the experimental data available.

1. The replacement of water by either organic solvents or D2O may alter the tertiary structure of the functional proteins of the respiratory chain, either by influencing the extent of their hydration by water, or by inducing changes in hydrogen bonding. The modification of tertiary protein structure in this manner may lead to an alteration in the spatial relationship of the respiratory chain components and thereby reduce the efficiency of their interaction.

2. Water may play a direct role in the transfer of reducing equivalents by participating in the protonation of an active group concomitant with the oxidation-reduction of cytochrome. This hypothesis recalls the suggestion made many years ago by Theorell (41, 49), and recently restated by Urry and Eyering (43), that hemoprotein electron transfer may occur through the resonating bonds of an imidazole ring. The replacement of water by either D2O or organic solvents, in the vicinity of the histidine groups which are postulated to participate in the transfer of reducing equivalents, would be expected to influence the course of the reaction. This hypothesis is similar to that of Laser and Slater (27), who explained the inhibition of succinate oxidation by D2O by postulating the existence of a rate-limiting protonolysis in the succinate oxidation system. The present data, which show that D2O inhibits at multiple sites in the respiratory chain, suggest that protonolytic processes may be involved in several reactions during the transfer of reducing equivalents from substrate to oxygen, and that a similar reaction mechanism is operative in each reaction. It would seem that the current concepts of respiratory chain reactions, as exemplified in the widely used terms "electron transfer" and "electron transport particle," may place undue emphasis on electron movements and ignore the possibility that hydrogen ions may be equally involved. However, the water content at the active sites of the respiratory chain reactions may be considerably lower than that in the bulk of the suspending fluid.

It is of interest that previous studies, which attributed the inhibition of enzymes by organic solvents to a reduction in water content (44, 45), also described marked inhibitions when the water content was reduced to about 50%. The use of nonaqueous solvents in the study of enzyme action remains a largely unexplored field, despite having some potential advantages over the use of aqueous media (45). It seems possible that the phenomenon described in the present paper is but one example of a more general requirement for the presence of water in efficient enzyme catalysis.

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