Studies on Choline Dehydrogenase*

I. EXTRACTION IN SOLUBLE FORM, ASSAY, AND SOME PROPERTIES OF THE ENZYME

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The majority of the dehydrogenases of mammalian mitochondria are linked to the respiratory chain (cytochrome system) by way of pyridine nucleotides. Although the intramitochondrial pyridine nucleotide appears to be tightly bound in intact mitochondrial preparations (1) and thus does not function as a mobile coenzyme, the requirement of these dehydrogenases for added dihydropyridine nucleotide or triphosphopyridine nucleotide is readily demonstrable after their removal from the mitochondrial environment. In contrast, the linkage of certain mitochondrial dehydrogenases to the respiratory chain does not appear to involve dissociable coenzymes even after extraction from the mitochondria and they were classified as "cytochrome-reducing" dehydrogenases in the older literature (2). In mammalian cells three dehydrogenases in this category have been recognized for a long time: succinic, α-glycerophosphate, and choline dehydrogenases. Besides the criteria mentioned they exhibit two further properties: lack of autooxidizability and resistance to extraction in soluble form. Since the identification of their prosthetic groups and the determination of their mechanism of action requires highly purified preparations free from other components of the respiratory chain, in 1954 our laboratory undertook the solubilization and isolation of these enzymes. After the isolation of succinic (3) and α-glycerophosphoric (4) dehydrogenases, the preparation of soluble choline dehydrogenase was undertaken as a first step in the isolation and characterization of the enzyme.

The extraction of choline dehydrogenase† in soluble form from acetone powders of rat liver mitochondria by means of cholate was claimed by Williams and Sreenivasan in 1953 (5). However, Ebisuzaki and Williams (6) later recognized that such preparations were dispersions of insoluble particles and that upon removal of the cholate the enzyme reverted to the insoluble form. They also surveyed a large variety of alternative methods for solubilization and found that of these only a combination of ultrasonic disintegration and treatment with n-butanol resulted in a soluble preparation (6). However, the very small (<10 per cent) yield in this procedure suggested that extensive inactivation had occurred. Thus it seemed desirable to devise an alternative and milder procedure for the extraction of the enzyme in good yield and active form before undertaking its isolation. In a preliminary note (7), we reported that the use of snake venom phospholipase A fulfills these criteria and that phenazine methosulfate is the only known electron acceptor suitable for the assay of the soluble enzyme. The present paper is a detailed account of this work.

EXPERIMENTAL

Materials and Methods

Rat liver mitochondria were isolated by the method of Schneider (8). Phospholipase D was purified from cabbage (9); crotoxin was a gift from Dr. H. Fraenkel-Conrat; and snake venoms were obtained from Ross Allen's Reptile Institute, Silver Springs, Florida. Choline chloride was purchased from the Nutritional Biochemicals Corporation, and its purity was established by nitrogen determination. The sources of the electron acceptors were as in previous studies (10, 11).

Routine assays of the enzyme were performed by the manometric phenazine methosulfate method (10) in the presence of 5 x 10⁻⁴ M phosphate, pH 7.8, 10⁻³ M cyanide, and 3.3 x 10⁻² M choline at 30 or 38°. All components except the dye were in the main compartment of Warburg vessels. After suitable temperature equilibration the contents of the side arm were tipped in and manometric changes were recorded for the period from 2 to 7 minutes after tipping. When precise activity determinations were desired, the dye concentration was varied and the results expressed as Vmax by the double reciprocal method. For routine work a dye concentration of 0.5 mg. per ml. was employed. All other assays were as previously described (10, 11).

In routine work the enzyme was extracted by the following procedure. Rat liver mitochondrial acetone powder was homogenized with 0.3 M phosphate buffer, pH 7.8, in the cold (50 ml. per gm. of acetone powder), and centrifuged for 25 minutes at 105,000 x g. The precipitate (Residue 1), containing all of the dehydrogenase initially present, was resuspended in 3 x 10⁻⁴ M Tris buffer, pH 7.4, and incubated with Naia naja venom for 40 to 60 minutes at 30°. The amount of venom to be used was determined for each batch of cobra venom and was usually about 10 mg. per gm. of protein in Residue 1. The suspension was

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† As used in this paper, the term "choline dehydrogenase" denotes the primary dehydrogenase which accepts electrons from the substrate; "choline oxidase" denotes the dehydrogenase and the components of the cytochrome chain which link NAD to O₂ in particulate preparations; and "choline-cytochrome c reduclease" is choline dehydrogenase plus all components of the respiratory chain which link NAD to externally added cytochrome c.
phenolindophenol presents a special problem, since the depend-
with the artificial acceptors are extrapolated velocities at infinite
dye concentration and are thus not influenced by inhibition at
methosulfate; the reaction rate with ferricyanide is still lower, and
ure only a part of the activity, as compared with phenazine
mentally the rates would be still lower. The use of 2,6-dichloro-
line oxidase assay) and with externally added cytochrome c meas-
ments are based on initial rates and were calculated as moles of
spectrophotometric; all others were manometric. All measure-
ments are based on initial rates and were calculated as moles of
substrate removed per unit of time. Rates are \( V_{\text{max}} \) except with
O\(_2\) and indophenol as acceptors (see text).

### Table I

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenazine methosulfate</td>
<td>100</td>
</tr>
<tr>
<td>( O_2 )</td>
<td>73</td>
</tr>
<tr>
<td>Cytochrome ( c )</td>
<td>63</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>42</td>
</tr>
<tr>
<td>2,6-Dichlorophenolindophenol</td>
<td>27</td>
</tr>
<tr>
<td>Methylen blue</td>
<td>4</td>
</tr>
</tbody>
</table>

Fig. 1. Variation of rate of choline oxidation with dye concen-
tration in the 2,6-dichlorophenolindophenol assay. Abscissa, re-
ciprocal dye concentration, in ml. of 0.02 per cent dye per ml. of
reaction mixture; ordinate, reciprocal velocity, expressed as \( \Delta E_{540} \)
per minute, corrected for endogenous blank rate. Conditions, as in “Materials and Methods,” except that temperature was 30°,
and 0.39 mg. of rat liver mitochondrial protein was present in each
cuvette. Dye reduction was followed in a recording spectropho-
tometer for 1 minute at various wave lengths to permit wide vari-
ation in dye concentration and calculated for 600 \( \mu \) (11).

Fig. 2. Variation of rate of choline oxidation with dye concen-
tration in the phenazine methosulfate assay. Abscissa, reciprocal dye concentration in ml. of 1 per cent dye per 3 ml. of reaction
mixture; ordinate, reciprocal velocity in moles of choline oxidized
per hour per mg. of protein. Standard assay at 30°; 7.8 mg. of
mitochondrial protein per vessel.
dehydrogenase with phenazine methosulfate is fully retained under these conditions, again in contrast to succinic dehydrogenase, which has two reaction sites with phenazine methosulfate in respiratory chain preparations, only one of which is retained on solubilization (19). 2,6-Dichlorophenolindophenol is the only other acceptor studied which is active in soluble preparations, but its relative activity has not been ascertained for the reasons mentioned above.

Since at very high concentrations of phenazine methosulfate the dye is inhibitory and since the rate varies measurably with dye concentration, extrapolation to \( V_{\text{max}} \) is recommended for the most accurate results. However, in routine assays at a fixed concentration of 0.6 mg. of dye per ml, a close approximation of the \( V_{\text{max}} \) may generally be obtained.

Preparation of Soluble Enzyme—The successful use of phospholipase A by Ringler and Singer (4) for the solubilization of the "cytochrome-reducing" \( \alpha \)-glycerophosphate dehydrogenase, an enzyme which resisted all other methods of extraction examined, suggested a study of this method for the solubilization of choline dehydrogenase. It became soon evident that under suitable conditions essentially complete extraction of the dehydrogenase could be obtained with either cobra venom (N. naja) or rattlesnake venom (Crotalus terrificus) as the source of phospholipase A. Typical experiments showing solubilization of the enzyme from fresh mitochondria and from acetone powders thereof are reproduced in Tables II and III, respectively. The use of acetone powder as a starting material offers a dual advantage. First, the enzyme may be converted to an acetone powder without loss of activity and is then very stable to storage; and second, conversion to acetone powder renders many of the proteins in rat liver mitochondria readily extractable, while choline dehydrogenase remains insoluble (5). Thus, preliminary extraction with phosphate buffer leaves behind a residue with much higher specific activity than the starting material, and on subsequent solubilization an 8- to 10-fold purification is achieved as compared with the original mitochondria, whereas solubilization of fresh mitochondria leads to only a 2- to 3-fold purification.

The extent of solubilization is a function of the nature of the buffer, ionic strength, the ratio of venom to mitochondrial protein, and time and temperature of incubation. The conditions listed in Table III were found to be best for rat liver mitochondrial acetone powders and N. naja venom. With other sources of the dehydrogenase and of the phospholipase, optimal conditions would have to be redetermined. Thus, with C. terrificus venom solubilization was nil at high (0.3 M) phosphate concentration, whereas with cobra venom this medium was not unsatisfactory (Table II). Mitochondria prepared in the presence of ethylenediaminetetraacetate should be freed from this reagent, as it inhibits the action of cobra venom phospholipase completely at a concentration of 10^{-4} M.

An increase in the total choline dehydrogenase activity of mitochondrial acetone powders upon extraction with phosphate buffer (Table III) was regularly encountered. The reason for this is not known, but it may be the consequence of the removal of inhibitory material. On the other hand, the partial inactivation upon incubation with snake venom (Table III), which did not occur if the venom was omitted from the incubation mixture, was often observed. In some instances, particularly with fresh mitochondria (Table II), little or no inactivation occurred.

The claim that treatment with snake venom results in soluble preparations of the dehydrogenase is based on the following observations. The resulting solutions were optically clear and remained so on extensive dialysis and repeated freezing and thawing, procedures which usually cause the reversal of extracts obtained by the use of surface active agents to the insoluble state. Upon ultracentrifugation at 144,000 \( \times \) g for 1 hour, 9 per cent of the activity was sedimented. The residue, resuspended in \( 3 \times 10^{-4} \) M Tris, pH 7.4, was incubated with 10 mg. of Naja naja venom per gm. of protein in Residue 1 at 30° for 40 minutes. After removal of an aliquot for assay the suspension was centrifuged for 25 minutes at 105,000 \( \times \) g. Assays as in Table II.

### Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total activity</th>
<th>( Q_{\text{m}} )</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh mitochondria</td>
<td>21,200</td>
<td>61.8</td>
<td>%</td>
</tr>
<tr>
<td>After 60 minutes of incubation with venom</td>
<td>23,200</td>
<td>65.4</td>
<td>100</td>
</tr>
<tr>
<td>Ultracentrifuged supernatant solution</td>
<td>23,300</td>
<td>149</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total activity</th>
<th>Total protein</th>
<th>( Q_{\text{m}} )</th>
<th>Yield</th>
<th>Solubilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial acetone powder</td>
<td>11,270</td>
<td>1,373</td>
<td>82.1</td>
<td>146</td>
<td></td>
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<tr>
<td>Same after phosphate extraction</td>
<td>16,510</td>
<td>377</td>
<td>438</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Suspension after incubation with venom</td>
<td>10,819</td>
<td>377</td>
<td>287</td>
<td></td>
<td>88 92</td>
</tr>
<tr>
<td>Supernatant solution after ultracentrifugation</td>
<td>9,943</td>
<td>163</td>
<td>610</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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**Table II**

**Solubilization of choline dehydrogenase from fresh mitochondria**

Conditions: Fresh rat liver mitochondria suspended in 0.3 M phosphate, pH 7.3, at a protein concentration of 22.1 mg. per ml. were treated with Naja naja venom (0.043 mg. per mg. of mitochondrial protein) for 1 hour at 30°. After removal of an aliquot for assay, the suspension was ultracentrifuged for 20 minutes at 144,000 \( \times \) g and the supernatant solution was reasayed (38°, phenazine methosulfate method).

**Table III**

**Solubilization of choline dehydrogenase from mitochondrial acetone powder**

Conditions: 2.9 gm. of rat liver mitochondrial acetone powder were extracted with 145 ml. of 0.3 M phosphate, pH 7.6, centrifuged at 105,000 \( \times \) g for 25 minutes, and the inactive extract was discarded. The residue, resuspended in 3 \( \times 10^{-4} \) M Tris, pH 7.4, was incubated with 10 mg. of Naja naja venom per gm. of protein in Residue 1 at 30° for 40 minutes. After removal of an aliquot for assay the suspension was centrifuged for 25 minutes at 105,000 \( \times \) g.
TABLE IV

Behavior of choline dehydrogenase in ultracentrifuge

Conditions: 7.7 ml. of an enzyme solution, isolated as described in Table III, were ultracentrifuged for 1 hour at 1° at 144,000 \( \times g \). The supernatant solution and the resuspended red-brown pellet were assayed as described in Table II, except that 30° was the temperature of the assay.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Distribution of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>%</td>
</tr>
<tr>
<td>Before ultracentrifugation</td>
<td>11,910</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant layer</td>
<td>9,910</td>
<td>84</td>
</tr>
<tr>
<td>Sedimented layer</td>
<td>1,991</td>
<td>9</td>
</tr>
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</table>

![Fig. 3: Variation of activity with pH. Abscissa, pH of reaction mixture at 38°; ordinate, ml. of O\(_2\) uptake per 5 minutes per ml. of enzyme in the standard phenazine methosulfate assay. Reaction period, 5 minutes; enzyme, soluble preparation, 16.4 mg. per ml.; O, in 5 \( \times 10^{-3} \) M phosphate; \( \bullet \), in 5 \( \times 10^{-3} \) M Tris plus 3 \( \times 10^{-2} \) M phosphate. The latter values are corrected for the slightly lower activity in Tris buffer.]

Stability—The dehydrogenase appears to be stable to storage in the cold for prolonged periods in mitochondrial acetone powders. Unlike succinic dehydrogenase (3), it is not significantly more stable in the particulate than in the soluble state. Thus, on 6 days of storage at -15°, pH 7.4, the loss of choline dehydrogenase activity (phenazine methosulfate assay) in mitochondria and in extracts was 20 and 13 per cent, respectively. Repeated freezing and thawing of the soluble enzyme appears to cause some inactivation. In one experiment 25 cycles of freezing and thawing resulted in 40 per cent inactivation, without precipitation of the enzyme. Heating of an extract for 3 minutes at 60° at neutral pH inactivate 63 per cent inactivation and cautious adjustment to pH 5 at 0° with acetic acid, followed by immediate neutralization, caused complete inactivation.

Effect of pH on Activity—At 30° the pH optimum is in the range of pH 7.6 to 8.2 in the phenazine methosulfate assay (Fig. 3) and the activity falls off rapidly on the acid side of this range. At pH 6.7 to 6.8, the value recommended by other investigators for the assay of the enzyme by different procedures (13, 22), only about 60 per cent of the activity is measured.

Effect of Substrate Concentration—In the phenazine methosulfate assay the \( K_m \) for choline is \( 7 \pm 1 \times 10^{-3} \) M at pH 7.6 and 38° (Fig. 4), which is significantly higher than the values recorded in the literature (1.2 to 2.9 \( \times 10^{-3} \) M at pH 7.4, 37°) (14, 23), in accord with the fact that in the present work the substrate concentration and not the reactivity with the electron carrier was the limiting factor.

Fig. 4. Variation of activity with choline concentration. Abscissa, reciprocal molarity of choline, average concentration during experiment; ordinate, reciprocal velocity in ml. per 5 minutes. Conditions: Standard assay at 38°, pH 7.6; soluble enzyme, 1.45 mg. of protein per vessel.

Cofactors—Both DPN and FAD have been reported to stimulate the oxidation of choline in rat liver mitochondria (15, 24). In the experiments of Strength et al. (24) the stimulatory effect of added DPN was demonstrated in mitochondrial preparations largely, but not entirely, freed from betaine aldehyde dehydrogenase. The effect was demonstrated as an increase in total O\(_2\) uptake in experiments of 60 minutes' duration. Since the extra O\(_2\) uptake elicited by added DPN exceeded that due to the oxidation of authentic betaine aldehyde, they reasoned that, although betaine aldehyde dehydrogenase is a DPN enzyme (25), the stimulation by DPN must have represented a direct effect of the coenzyme on choline oxidase.

When the kinetics of the DPN effect on choline oxidase are examined (Fig. 5), it becomes evident that the increase in the rate of O\(_2\) uptake with added DPN is initially almost negligible and increases with time and, further, that it represents primarily a maintenance of the initial rate in the DPN samples, whereas the controls decline gradually. Since betaine aldehyde is a powerful inhibitor of choline dehydrogenase (26) whereas its oxidation product, betaine, is not, the stimulation of choline oxidase may now be explained as an activation of betaine aldehyde de-
hydrogenase, resulting in (a) increased O₂ uptake owing to oxidation of the aldehyde, and (b) removal of the inhibitory aldehyde and consequent protection of choline dehydrogenase. Soluble choline dehydrogenase, which is free from the aldehyde dehydrogenase, can neither reduce DPN with choline as a substrate nor oxidize DPNH with betaine aldehyde as electron donor.

FAD at high concentrations and in the presence of Mg⁺⁺ has been reported to stimulate the choline-cytochrome c reaction in mitochondria which had lost most of their choline-cytochrome c reductase activity on extensive dialysis against 0.25 M sucrose (15). As documented elsewhere (27), an attempt to repeat these experiments was unsuccessful: dialysis resulted in a negligible decline in choline-cytochrome c reductase and FAD ± Mg⁺⁺ failed to stimulate the activity of either dialyzed mitochondria or of purified soluble preparations. It may be concluded that choline dehydrogenase requires no readily dissociable coenzyme for activity, but the results have no bearing on the possible presence of tightly bound coenzymes.

Other Properties—Other characteristics of the enzyme relevant to the determination of its activity are as follows. Unlike the succinic dehydrogenase of animal tissues (28), which it resembles in many other respects, choline dehydrogenase does not appear to be activated by the substrate. At 30 or 38°C the reaction rate declines much faster in the choline dehydrogenase (phenazine methosulfate) assay than in the choline oxidase assay, as expected from the facts that choline dehydrogenase is an —SH enzyme (29) and that the cyanide present in the reaction mixture offers only incomplete protection against the action of the H₂O₂ produced in the reoxidation of phenazine methosulfate (10). Consequently, a short (5 minute) assay period is recommended.

Mitochondrial preparations oxidize choline by way of the respiratory chain equally well with or without added cytochrome c, although succinate oxidation by the particles is greatly stimulated by supplementation with cytochrome c (Table V).

**DISCUSSION**

As in the case of succinic dehydrogenase, successful solubilization of choline dehydrogenase required a reliable assay method for the primary dehydrogenase and a technique for liberating the enzyme from the lipoproteins to which it appears to be bound in mitochondria, without attendant inactivation. Like the succinic and α-glycerophosphate dehydrogenases of animal tissues (4, 17), soluble preparations of choline dehydrogenase, separated from the cytochrome chain, react best with phenazine methosulfate as acceptor. Unlike succinic dehydrogenase, however, which retains one of its reaction sites with ferriyanide on solubilization (19) and has a broad specificity for electron donors in the direction of fumarate reduction, which may be employed in its assay (18), and unlike α-glycerophosphate dehydrogenase which retains its full activity toward ferriyanide in soluble preparations, choline dehydrogenase does not react with ferriyanide in soluble preparations and so far no satisfactory electron donor has been found to follow its possible activity in the direction of betaine aldehyde reduction. Since, for the reasons set forth in this paper, the indophenol assay is not considered reliable, as pointed out earlier (17) and recently confirmed by Korzenovsky and Auda (30), at present the phenazine methosulfate provides the only known satisfactory assay for choline dehydrogenase.

The loss of reactivity with ferriyanide appears to be a direct result of the separation of the dehydrogenase from the respiratory chain, since solubilization of the enzyme from fresh mitochondria with phospholipase A is attended by immediate loss of the choline-ferricyanide (and of the choline-cytochrome c) reactions. Alternate methods of extraction also entail complete loss of activity toward ferricyanide (30), and, in fact, it has been claimed that a separate enzymatic component is required for linking choline dehydrogenase to ferricyanide (31), similarly to the choline dehydrogenase-neotetrazolium reaction (32). If such a factor indeed exists, it would have to intercept electron transport below the level of cytochrome c, since in mitochondria the choline-ferricyanide reaction is insensitive to antimony A, whereas the choline-cytochrome c reaction is abolished by this reagent (26).

In regard to methods which lead to extraction of the enzyme in soluble form, choline dehydrogenase differs sharply from succinic dehydrogenase, which is readily extractable from solvent-extracted mitochondria with alkaline buffers (17, 33), and it resembles α-glycerophosphate dehydrogenase, which is not extracted by this treatment but is readily liberated under the influence of phospholipase A (4). Shortly after the authors' note on the preparation of soluble choline dehydrogenase was submitted for publication there appeared a report on the solubilization of choline dehydrogenase from rat liver by the use of the nonionic detergent isooctylphenoxypolyethoxylate (30). The enzyme extracted by this method is reported not to sediment in 1 hour at 144,000 × g. It remains to be determined whether the enzyme in such extracts is in true solution and amenable to purification, however, since detergents are known to form very stable complexes with proteins, which do not readily dissociate on dialysis, and do not sediment in high centrifugal fields but, at least in some cases, prevent the fractionation of proteins by conventional methods.

The effectiveness of phospholipase A in liberating choline dehydrogenase in soluble form, as compared with numerous other methods (5, 6), lends considerable support to the hypothesis that the cytochrome-reducing dehydrogenases are linked to the cytochrome chain by way of phospholipide bridges. A closer study of the specificities of the phospholipases effective in solubilizing the enzyme may lead to a clue to the mode of attachment of the enzyme to the cytochrome chain. In preliminary studies it has already been ascertained that phospholipase D, which liberates the nitrogenous base in phospholipides, and the phospholipase A of Crotalus adamanteus, which acts on the same bond as the phospholipase A of N. naja venom employed here, but has a different specificity toward the fatty acid residues (34), are completely ineffective in solubilizing choline dehydrogenase.

### Table V

**Effect of added cytochrome c on succinoxidase and choline oxidase activities of rat liver mitochondria**

<table>
<thead>
<tr>
<th>Additions</th>
<th>O₂ uptake (μl)</th>
<th>Q₀ (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>15.6</td>
<td>24</td>
</tr>
<tr>
<td>Choline + cytochrome c</td>
<td>17.0</td>
<td>26</td>
</tr>
<tr>
<td>Succinate</td>
<td>36.4</td>
<td>55</td>
</tr>
<tr>
<td>Succinate + cytochrome c</td>
<td>119.0</td>
<td>183</td>
</tr>
</tbody>
</table>

*Conditions: Rat liver mitochondria, isolated in 0.25 M sucrose, 7.5 mg per vessel; 5 × 10⁻⁵ M phosphate, pH 7.6; 3.3 × 10⁻⁴ M choline or 2 × 10⁻⁴ M succinate; cytochrome c, where added, 1.25 μg; total volume, 3 ml; temperature, 30°; reaction period, 5 minutes.*
In view of the probability that the active component of snake venoms in solubilizing choline dehydrogenase is phospholipase A, the observation of Braganza and Quastel (35) that fresh as well as boiled cobra venom rapidly inactivates "choline oxidase" may be clarified. The authors interpreted their results as indicating that the thermostable phospholipase A was the agent responsible for the inactivation and that the latter was due to the "enzymic degradation of mitochondrial structures upon whose integrity or particular spatial configuration the enzyme systems...depend for their optimal activities" (35). As already discussed, the action of cobra venom phospholipase A effectively abolishes the choline dehydrogenase-ferricyanide reaction without inactivating the dehydrogenase itself (Table II). Therefore, in the main, the conclusions of Braganza and Quastel are correct, except that the phospholipase A interrupts only the flow of electrons to the electron transport system but does not affect the primary dehydrogenase.

**SUMMARY**

1. The assay of choline dehydrogenase activity in mitochondrial as well as soluble preparations with phenazine methosulfate as acceptor has been described. Electron acceptors employed in the past for the assay of this enzyme (cytochrome c, ferricyanide, methylene blue, and 9,6-dichlorophenolindophenol) either do not react directly with the dehydrogenase and consequently measure a variable part of its activity, or they are unreliable because of the extensive dependence of the measured activity on dye concentration.

2. Choline dehydrogenase has been brought into solution in good yield by a mild procedure involving treatment of mitochondria or acetone powders thereof with certain snake venoms. Evidence has been presented that the active component in liberating the dehydrogenase in soluble form is phospholipase A.

3. The process appears to depend on the point of attack and on the structural specificity of the particular phospholipase, since plant phospholipase D and the phospholipase A of certain venoms are inactive, whereas the phospholipase A of other venoms is active in dissolving the dehydrogenase.

4. Upon treatment with phospholipase A the reactions of the dehydrogenase with cytochrome c and ferricyanide are abolished, probably as a result of the uncoiling of the enzyme from the factors linking it to these acceptors in respiratory chain preparations.

5. The enzyme extracted by this means does not sediment at 144,000 x g for 1 hour and does not precipitate on repeated freezing and thawing or on prolonged dialysis.

6. Contrary to claims in the literature, choline dehydrogenase is not stimulated by flavin adenine dinucleotide or diphosphopyridine nucleotide, even after extensive dialysis, in either soluble or mitochondrial preparations. The apparent stimulation of the dehydrogenase by added diphosphopyridine nucleotide in assays involving the respiratory chain (choline oxidase) has been explained as an indirect effect involving the activation of bovine aldehyde dehydrogenase, with consequently increased rate of O_2 uptake and removal of the aldehyde which is inhibitory to choline dehydrogenase.

7. Some properties of the soluble dehydrogenase have been described.

**REFERENCES**

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George Rendina and Thomas P. Singer