A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF CHOLINE DEHYDROGENASE*

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(Received for publication, October 7, 1953)

The increased oxygen uptake of liver suspensions on the addition of acetylcholine, which was reported by Bernheim and Bernheim in 1933 (2), was shown by Mann and Quastel (3) to be due to the oxidation of choline. Betaine aldehyde was identified as the oxidation product (3). The choline oxidase (CO) system was found by Mann, Woodward, and Quastel (4) to consist partly of a typical dehydrogenase. They demonstrated that, in the presence of choline, rat liver extracts reduced sodium ferricyanide anaerobically and cytochrome c aerobically and concluded that the CO system consisted of choline dehydrogenase (CD), cytochrome c, and cytochrome oxidase. During the course of developing the procedure presented in this paper, it was found (1) that the antimycin-sensitive factor of the succinoxidase and diphosphopyridine nucleotide (DPN) cytochrome c reductase systems (5, 6) was involved in the oxidation of choline by rat liver and kidney. This was confirmed by Ebisuzaki and Williams in a subsequent report (7). Evidence was also obtained (1) implicating the Slater factor (8), believed to be a component of the succinoxidase system required for the transmission of electrons between cytochrome b and cytochrome c, in the CO system. Since both BAL and antimycin inhibit CO, and since the inhibition is reversed by artificial carriers such as methylene blue and brilliant cresyl blue, it is possible that the two compounds are acting on the same substance, although this has yet to be proved. It should be noted, as pointed out by Edelhoch et al. (9), that the lack of effect of antimycin on a soluble DPN cytochrome c reductase (9) may indicate that the antibiotic acts on some site in particulate preparations which affects reductase activity and not on the reductase protein per se. While no other component of the CO system has been unequivocally identified, riboflavin (10, 11) in the form of flavin adenine dinucleotide (7), DPN (12–14), folic acid, Leuconostoc citrovorum factor, vitamin B12, and ascorbic acid (15–18) have all been shown to enhance the activity of liver CO. A soluble CD

* A preliminary report of this work has appeared (1).
1 Eichel, H. J., unpublished data.
has been prepared recently by extraction of liver mitochondria with sodium cholate (19).

Several methods have been employed for the study of choline oxidation in animal tissues. One of these depends upon the use of ferricyanide as a hydrogen acceptor (4). The reaction is followed by measuring the carbon dioxide displaced from a bicarbonate medium under anaerobic conditions. Bargoni and DiBella (20) used the Thunberg technique with methylene blue as a hydrogen acceptor in their investigation of the effects of various agents on CD. More recently, Williams, Litwack, and Elvehjem (21) described a manometric assay for CO based on following oxygen consumption. Preparatory to initiating a program aimed at the isolation and purification of CD, it was felt that a spectrophotometric procedure for assay of the enzyme would have several advantages over the manometric method generally employed; it would eliminate from the test system at least one enzyme, namely cytochrome oxidase, require far less protein, and save time during the course of routine assays of many enzyme samples. In addition, investigation of the effect of activators or inhibitors on CD can be studied under less complicated conditions but in the presence of a physiological electron carrier. This paper presents a spectrophotometric method for the determination of CD activity in homogenates of liver and kidney, as well as some observations on the properties of the enzyme.

**Methods and Materials**

*Enzyme Source*—Male Wistar rats, weighing from 150 to 300 gm. and maintained on a diet of fox chow, were used in this study. Livers and kidneys were homogenized with a ground glass homogenizer in cold 0.039 M Na₂HPO₄-KH₂PO₄ buffer, pH 6.8.

*Assay of Choline Dehydrogenase and Calculations*—The rate of reduction of cytochrome c was followed in a Beckman spectrophotometer (model DU) at 550 μm with a slit width of 0.025 mm. The instrument was fitted with a block in which water circulated from a constant temperature bath maintained at 25° ± 0.05°. All reactants, except the homogenates which were immersed in ice, were kept at 25° in the water bath. Zero time was taken as the time of inversion of the covered cuvette immediately after the addition of cytochrome c. Subsequent time values were taken at densities 0.010 to 0.025 above the initial one, until at least 2½ minutes had elapsed or the oxidized cytochrome c had become nearly reduced. At the end of this time a few grains of Na₂S₂O₄ were added and the extinction determined to give the fully reduced value. The concentration of oxidized cytochrome c present at any time was calculated from the equation

\[
[Ferricytochrome c] = \frac{(D_t - D_{\infty})}{E_o - E_r} l
\]
where $D_\infty$ was the density at infinite time (the fully reduced value), $D_t$ was the reading at any time $t$ during the course of the reaction, $E_0$ and $E_r$ the molar extinction coefficients for oxidized and reduced cytochrome $c$ at 550 m\(\mu\) (22), and $l$ the length of the cell in cm. If the [ferricytochrome $c$] was plotted against time for a series of six or more readings, a straight line was obtained. The $k$ value (reaction rate constant) was calculated directly from the slope of the line and was expressed as micromoles of cytochrome $c$ reduced per minute. The specific activity was obtained by dividing the $k$ value by the mg. of total nitrogen in the homogenate.

Beef cytochrome $c$, obtained from Wyeth, Incorporated, was generally found to be over 90 per cent pure as determined from its extinction coefficient at 550 m\(\mu\) (22). It was assumed to have a molecular weight of 12,350 (23). DPN (90 per cent pure) was purchased from the Sigma Chemical Company. Choline chloride and nicotinamide were obtained commercially.

Nitrogen determinations were performed by a micro-Kjeldahl procedure and manometric measurements of CO activity were carried out by the method of Williams et al. (21).

**RESULTS AND DISCUSSION**

The details of the procedure finally adopted will be presented first and the effect of varying the concentrations of each of the components, with liver homogenates, will follow.

**Final Assay System**—For optimal CD activity, the following reactants were added to the cuvette, in the following order: 0.1 ml. of 0.72 \(M\) choline chloride, a suitable amount of homogenate (usually 0.1 ml. of a 1:24 dilution), 0.1 ml. of 0.03 \(M\) NaCN, 1.0 ml. of 0.2 \(M\) Na$_2$HPO$_4$-KH$_2$PO$_4$ buffer, pH 6.8, distilled water to make a volume of 3.0 ml., and 2.7 $\times$ 10$^{-5}$ \(M\) cytochrome $c$. The blank contained distilled water. A control containing all the components except choline was necessary, since at the dilutions used both liver and kidney homogenates were capable of reducing cytochrome $c$ in the absence of added substrate. This endogenous reduction of cytochrome $c$ was always subtracted from the activity obtained in the presence of choline.

**Concentration of Tissue**—Fig. 1, a shows the rate of reduction of cytochrome $c$ by varying amounts of liver homogenate in the complete system described above. It can be seen that the reaction proceeded linearly for several minutes. Fig. 1, b indicates that the activity was directly proportional to the concentration of tissue. Samples of kidney homogenates gave similar results.

**Substrate Concentration**—The effect of varying concentrations of choline chloride on CD activity is shown in Fig. 2. With 0.1 ml. of a 1:24 liver
homogenate, there was little change in activity over the range 7.2 to 72 
$\mu$M of choline. An increase in the substrate concentration markedly in-
hhibited the enzyme; this is in agreement with data reported previously for 
the manometric determination of CO (21).

Cytochrome $c$ Concentration—The following data indicate that in the final 
assay system the reaction is zero order with respect to the concentration of 
cytochrome $c$. At 0.68, 1.4, 2.7, and $5.4 \times 10^{-5}$ M cytochrome $c$, the $k$
values were 0.84, 0.93, 0.99, and $1.02 \times 10^{-2}$, respectively.

![Graph](http://www.jbc.org/)

**Fig. 1.** Choline dehydrogenase activity of rat liver homogenate. a, reduction of 
cytochrome $c$ by 0.05 (●), 0.1 (○), and 0.2 (△) ml. of homogenate as a function of 
time; b, activity as a function of tissue concentration. The homogenate dilution 
was 1:24. For the components of the reaction mixture, see the text.

**Phosphate Buffer**—At pH 7.4 in the presence of choline, the reaction rate 
was essentially independent of the concentration of Na$_2$HPO$_4$-KH$_2$PO$_4$
buffer from 0.0033 to 0.067 M. Since the reduction of cytochrome $c$ by 

liver and kidney in the absence of added choline was routinely subtracted 
from the activity obtained in the presence of added choline, the effect of 
phosphate concentration was studied on the endogenous activity. The 
latter was found to be markedly dependent on the concentration of phos-
phate buffer. Activity was optimal from 0.013 to 0.025 M and decreased 
significantly with increasing phosphate concentrations. For six different 
liver homogenates, the activity at 0.013 M phosphate was 114 per cent 
higher than that obtained at 0.067 M phosphate. Hence, all enzyme assays 
were performed at a final phosphate concentration of 0.067 M. The mean 
$k$ value for liver in the absence of choline was $0.12 \times 10^{-2}$ (0.06 to 0.17), 
while for kidney this value was $0.09 \times 10^{-2}$ (0.06 to 0.15).
**Effect of pH**—In this study, attention was focused on pH 6.8, 7.4, and 7.8, since Bernheim and Bernheim (24) had shown that at pH 6.7 choline is oxidized to betaine aldehyde and at pH 7.8 to betaine. Williams et al. (21) recently reported that there was no significant difference in CO activity when the enzyme was studied manometrically at pH 6.8, 7.3, and 7.8. In Fig. 3 it can be seen that the optimal pH for CD activity occurred at 6.8. The activity was diminished by almost 50 per cent at pH 7.8; at pH 6.15, it was still 80 per cent of the maximum. When CO was assayed manometrically in the presence of 0.013 M or 0.067 M phosphate buffer it was found that for the first 10 minute period following equilibration the
activity at pH 7.8 was only 60 to 70 per cent of that at pH 6.8. This result is in agreement with the spectrophotometric data presented above. At 0.067 M phosphate in the absence of added choline, it was observed that the rate of reduction of cytochrome c increased as the pH was increased from 6.8 to 7.8.

Effect of DPN and Nicotinamide—Although it has been recently stated that CD is a DPN-linked enzyme (14), no consistent effect of DPN on CD activity could be demonstrated either in the presence or absence of nicotinamide at pH 6.95, 7.4, or 7.8. Nicotinamide alone has been reported to inhibit the rat liver CO system strongly (21), while Colter and Quastel (25) observed a negligible reduction of rat liver CO in the presence of 74 \mu M of nicotinamide. Doctor et al. (26) found that 143 \mu M of this substance had no effect on chick liver CO. Table I summarizes the effect of nicotinamide on the CD activity of rat liver. The data indicate that the enzyme is somewhat inhibited at relatively high concentrations of nicotinamide.

Normal Values—CD activities of homogenates prepared from the livers and kidneys of normal animals are presented in Table II. For comparable animals, the activity of kidney was more constant than that of liver. It is of interest to compare quantitatively the CD values obtained spectrophotometrically with the CO activities measured manometrically, although it is realized that our knowledge of the various factors involved in each of the tests is still incomplete; i.e., the question of the catalytic activity of endogenous cytochrome c versus exogenous cytochrome c, the possibility of dilution effects on different components, etc. Since the oxygen consumption observed in the manometric assay for CO is presumably the end-result of electron transfer via cytochrome c, and since cytochrome oxidase is probably present in wide excess over the dehydrogenase in both tissues, it holds that the rate of reduction of cytochrome c should limit the rate of oxygen uptake. Hence, the activity of the CO system as measured by the

<table>
<thead>
<tr>
<th>Nicotinamide (\mu M)</th>
<th>Inhibition per cent</th>
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<tbody>
<tr>
<td>24.6</td>
<td>0</td>
</tr>
<tr>
<td>32.8</td>
<td>5</td>
</tr>
<tr>
<td>49.2</td>
<td>9</td>
</tr>
<tr>
<td>82.0</td>
<td>15</td>
</tr>
<tr>
<td>131.2</td>
<td>17</td>
</tr>
</tbody>
</table>
Warburg technique should be comparable to that of the CD system determined spectrophotometrically, provided that (a) the same enzyme or enzyme complex is measured by the two methods and (b) optimal considerations for substrate concentration, pH, etc., have been met in each case. Calculation of the activity of kidney CO required to reoxidize cytochrome \( c \) at the same rate that it was reduced by kidney CD (1 mole of oxygen being equivalent to 4 moles of reduced cytochrome \( c \)) yields a \( Q_{o2} \) (N) (c.mm. of oxygen consumed per mg. of N per hour) of 38.8. This figure is in good agreement with the experimentally determined \( Q_{o2} \) (N) of 45.5 (see Table III) for kidney CO. In the case of liver, however, the observed reduction of cytochrome \( c \) by CD occurs at less than half the rate at which the liver CO system was found to consume oxygen. While the \( Q_{o2} \) (N) for liver CO required to keep pace with liver CD was calculated to be 23.2,

**Table II**

*Choline Dehydrogenase Activity of Rat Liver and Kidney Homogenates*

The values represent the average and standard deviation and have been corrected for the reduction of cytochrome \( c \) by homogenate in the absence of added choline.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of rats</th>
<th>( \times 10^{-3} )</th>
<th>Specific activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>23</td>
<td>0.82 ± 0.25</td>
<td>0.075 ± 0.020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.63 - 1.26)</td>
<td>(0.048 - 0.113)</td>
</tr>
<tr>
<td>Kidney</td>
<td>13</td>
<td>1.19 ± 0.18</td>
<td>0.105 ± 0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.90 - 1.44)</td>
<td>(0.073 - 0.134)</td>
</tr>
</tbody>
</table>

* Micromoles of cytochrome \( c \) reduced per minute.
† Micromoles of cytochrome \( c \) reduced per minute per mg. of nitrogen.

the experimentally measured value was 52.2 (Table III). Accordingly, several efforts were made to enhance the activity of liver dehydrogenase. It has been reported that succinic dehydrogenase activity can be increased by homogenizing in the presence of succinate (27). However, no increase in CD activity was observed after homogenizing with choline. The possibility was considered that betaine aldehyde, produced by the dehydrogenation of choline, was reacting with the sodium cyanide and preventing the complete inhibition of cytochrome oxidase. A 10-fold increase in the final concentration of cyanide (0.01 M) had little effect on the reaction rate.

It should be noted that in several cases the CD activity of liver homogenates approached the theoretical value calculated on the basis of manometric data. These results, and the large number of values presented in Table II, may possibly be explained by the fact that one of the cofactors of the CD system may be stored to different degrees in the livers of different animals. If the level of this factor should be reduced to a point where
it becomes rate-limiting at the 1:720 tissue dilution employed in the cuvette, the CD value would be lowered accordingly. This problem is now under investigation.

Effect of Temperature on CO—In the course of determining CO activity in liver and kidney homogenates under various experimental conditions, it was observed that at 35° the activity of the liver enzyme was almost twice that of kidney. Bernheim and Bernheim (24), Kensler et al. (28), and Richert and Westerfeld (29) also reported this ratio at 37°. However, at 25° under the conditions used in this laboratory, liver CO activity was only slightly greater than that of kidney. Table III lists the activities of liver and kidney CO at 25° and 35°. Since assays were not performed in every instance on the same tissue sample at the two temperatures, this is not to be considered a study of the temperature coefficients of liver and kidney CO. However, from the ratios of activity at the two temperatures, it would appear that the Q₁₀ of the liver enzyme is markedly higher than that of kidney. This relationship is constant whether the activity is expressed on a wet weight or N basis. It is to be noted that Doctor et al. (26) and Dinning et al. (30) found little difference between the activities of chick liver and kidney CO at 37°.

### Table III

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Temperature °C</th>
<th>No. of rats</th>
<th>O₂ per 167 mg. wet weight per 10 min.</th>
<th>Q₁₀ (N)</th>
<th>Ratio 35°/25°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>35</td>
<td>69</td>
<td>70.3 (52-96)</td>
<td>88.3</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>10</td>
<td>40.5 (35-54)</td>
<td>52.2</td>
<td>1.69</td>
</tr>
<tr>
<td>Kidney</td>
<td>35</td>
<td>15</td>
<td>37.0 (29-50)</td>
<td>51.8</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>10</td>
<td>30.5 (21-38)</td>
<td>45.5</td>
<td>1.14</td>
</tr>
</tbody>
</table>

SUMMARY

A spectrophotometric method is presented for the assay of choline dehydrogenase activity in homogenates of rat liver and kidney. The method depends upon measurement of the rate of reduction of cytochrome c. Optimal activity is obtained at pH 6.8 and at a final phosphate concentration of 0.067 M. The addition of DPN had no effect on the reaction rate...
while nicotinamide inhibited the reduction of cytochrome c slightly. Data are given for the content of the enzyme in rat liver and kidney and are discussed in comparison with choline oxidase values of the two tissues obtained by manometric measurement. Liver choline oxidase appears to have a higher temperature coefficient than that of kidney over the range 25–35°.

Addendum—After the completion of this investigation, Williams and Sreenivasan (19) reported a spectrophotometric method for the determination of choline dehydrogenase which by-passes the cytochrome system by using 2,6-dichlorophenol indophenol as a hydrogen acceptor.

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