

PREPARATION OF SOLUBLE CHOLINE DEHYDROGENASE FROM LIVER MITOCHONDRIA*

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Recently it has been demonstrated that the choline oxidase system is located in the mitochondrial fraction of rat liver (1, 2). Because of the insolubility of the proteins of liver mitochondria, very few of the enzymes in this liver fraction have been prepared in soluble form. Using sodium desoxycholate as a dispersing agent, Eichel *et al.* (3) have reported the preparation of a soluble cytochrome oxidase from the insoluble fractions of heart. Drysdale and Lardy (4) have recently succeeded in preparing a soluble fatty acid dehydrogenase from rat liver acetone powder.

In a previous report from this laboratory (5) it was indicated that attempts to extract the choline oxidase system from isolated mitochondria with sodium choleate as a dispersing agent failed. At that time, however, choline oxidase activity was measured manometrically with oxygen as a hydrogen acceptor. Therefore, the entire mitochondrial hydrogen transport system, including the cytochromes (6), would have been required to detect choline oxidase activity. If any one of the hydrogen transport components was not extracted by sodium choleate, no activity would have been obtained. In the present report we have reinvestigated this problem with a spectrophotometric method for following choline dehydrogenase activity in which the cytochrome system is not required. If choline dehydrogenase can be prepared in a soluble form, studies of its cofactors, mechanism of action, and other properties which have hitherto been investigated only in crude preparations (5, 7-13) could be greatly advanced.

EXPERIMENTAL

Development of Assay Method—To initiate these studies, a general method for following choline dehydrogenase activity spectrophotometrically was developed. A variety of hydrogen acceptors which give a change in optical density when reduced was studied. The acceptor best suited for this method was found to be 2,6-dichlorophenol indophenol, since it has a maximum at 607 m μ which disappears when it is reduced.

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Mitochondria were isolated from livers of adult male rats of the Sprague-Dawley strain by methods previously described (5). In preliminary experiments with 2,6-dichlorophenol indophenol as hydrogen acceptor, the washed mitochondria from 4 gm. of liver were suspended in 25 ml. of 0.1 M sodium phosphate buffer (pH 7.3). The standard assay system was prepared as follows: 0.6 ml. of 10 mg. per cent 2,6-dichlorophenol indophenol, 0.6 ml. of Krebs-Ringer-phosphate buffer (pH 7.3) (14), 0.8 ml. of water, and 0.2 ml. of 2 per cent choline chloride or water (for the control) were mixed in test-tubes. The mixture was transferred to the cuvette of the spectrophotometer (Beckman quartz spectrophotometer, model DU) and 1 ml. of the mitochondrial suspension directly pipetted into the mix-

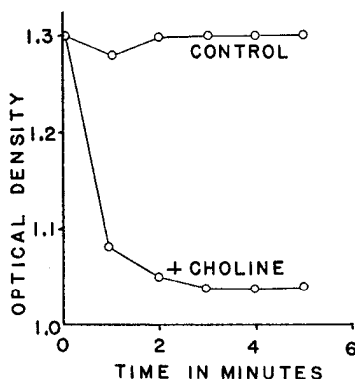


FIG. 1. Choline dehydrogenase activity of fresh whole mitochondria with 2,6-dichlorophenol indophenol as the hydrogen acceptor (Beckman model DU, 607 $m\mu$).

ture. The cuvette was quickly inverted to mix the enzymes with the other components and was placed in the spectrophotometer chamber. Readings of optical density at 607 $m\mu$ were taken at 1 minute intervals for 5 minutes. The results of a typical experiment of this type are presented in Fig. 1. The optical density of the mixture containing choline rapidly decreased to a constant value while the control mixture, containing no choline, gave little decrease in optical density. As will be seen in later experiments, the control curve oftentimes gave some reduction of the dye, probably because of the presence of endogenous metabolites. In every case, however, the rate of decrease of optical density was greater in the mixture containing choline.

From curves similar to those given in Fig. 1, the authors have developed an arbitrary method for calculating the enzyme activity which can be expressed by a number rather than a graph. The optical density value of the points of each curve is subtracted from the immediately preceding

value. The figures thus obtained for the control system are then subtracted from the corresponding values of the system containing choline. The maximal difference in change in optical density is then used to express choline dehydrogenase activity. Thus, in Fig. 1, the activity was calculated to be 200 units of change in optical density \times 1000 per minute. In this example the maximal difference in change in optical density was during the first minute.

To obtain the optimal concentration of dye for the system, experiments were carried out in which the level of dye added to the system was varied over a range from 10 to 100 γ per cuvette. Other components of the system were kept at the same concentrations as listed above. The results of these experiments, in which the choline dehydrogenase activity is expressed as maximal change in optical density \times 1000 per minute between the control and the system containing choline, are summarized as follows: 20 γ of dye 132, 40 γ of dye 152, 60 γ of dye 210, 80 γ of dye 250, and 100 γ of dye 240. Thus the optimal dye concentration for this system was about 80 γ of dye per cuvette.

Further Purification of System—In the recent work of Drysdale and Lardy (4), rat liver mitochondria were treated with acetone to prepare them for the aqueous extraction of hitherto insoluble fatty acid dehydrogenase. The treatment with acetone allowed the enzyme to be extracted with either water or dilute buffer. To observe whether extraction of choline dehydrogenase from mitochondria could be achieved in a similar manner, mitochondria were isolated from rat liver in isotonic sucrose as before. The twice washed mitochondria were homogenized with 20 volumes of cold acetone and centrifuged. The acetone treatment was repeated. The residue was taken up in 20 volumes of cold diethyl ether (c.p.) and recentrifuged. The mitochondria were then partially air-dried on filter paper for 2 to 3 minutes, and the last traces of ether were removed *in vacuo*. This powder was found to be stable with respect to choline dehydrogenase activity for at least 2 weeks at -5° . A yield of about 4.0 to 4.5 gm. of dried mitochondria per 100 gm. of fresh rat liver was obtained.

The choline dehydrogenase activity of the acetone-treated mitochondria was assayed by homogenizing the powder in 0.1 M sodium phosphate buffer (20 mg. of powder per ml. of buffer). When 1 ml. of the homogenized mitochondrial suspensions was used in a total assay volume of 3.2 ml. with the same component concentrations as in the preceding section, curves similar to those of Fig. 1 were obtained, indicating that the acetone-ether treatment did not destroy the choline dehydrogenase activity. By using this system, it was found that the optimal choline chloride concentration was 4 mg. per cuvette, which fortuitously was the same concentration as that used in the preceding work with the untreated fresh mitochondria.

The pH optimum of the system with acetone-treated mitochondria was found to be 6.8 rather than 7.3. Therefore, other solutions were brought to pH 6.8.

Preparation of Soluble Choline Dehydrogenase—First attempts to bring choline dehydrogenase into aqueous solution from acetone-ether-treated mitochondria indicated that the enzyme could not be extracted with either cold distilled water or 0.1 M sodium phosphate buffer (pH 6.8). Homogenization of the dried mitochondria with water or buffer and subsequent centrifugation gave no activity in the supernatant layer, but all of the activity was recovered in the residue. Thus the solubility of choline dehydrogenase is different from that of mitochondrial fatty acid dehydrogenase since the latter system can be extracted from acetone-dried mitochondria with either water or buffer (4).

When the buffer suspension of the dried mitochondria was frozen in a mixture of dry ice and acetone and rapidly thawed at 37°, no choline dehydrogenase activity was obtained in the supernatant layer after centrifuging, but the activity was recovered completely in the insoluble residue.

Because of the dispersive action of bile salts on insoluble lipides, the effect of sodium choleate (Merck) on the solubility of choline dehydrogenase of acetone-ether-dried mitochondria was studied. A mixture of 20 mg. of dried mitochondria and 12 mg. of sodium choleate (pH 6.8) (added in concentrated solution) per ml. of 0.1 M sodium phosphate buffer (pH 6.8) was homogenized in a glass homogenizer for 1 to 2 minutes and centrifuged at $25,000 \times g$ for 30 minutes. A control suspension of the mitochondria was prepared in the same way, except that the sodium choleate was omitted. The supernatant layers of both mixtures were decanted and the residues resuspended in 1 ml. of buffer per 20 mg. of original mitochondria. The choline dehydrogenase activities of 1 ml. aliquots of the supernatant solutions and resuspended residues of both preparations were then assayed with the optimal 2,6-dichlorophenol indophenol level (80 γ), choline chloride (4 mg.), and Krebs-Ringer-phosphate buffer (pH 6.8). The final volume in each cuvette was 3.2 ml. The results of a typical experiment of this type are presented in Fig. 2. From the extraction with buffer alone, all of the activity still resided in the insoluble mitochondrial residue. However, when sodium choleate was included in the extraction mixture, all of the choline dehydrogenase activity was brought into the supernatant solution with no activity remaining in the insoluble fraction. The supernatant layer was absolutely clear and, even after centrifuging for 60 minutes at $28,000 \times g$, no activity was lost from the solution and no increase in activity in the residue was obtained.

To obtain the optimal concentration of sodium choleate for extracting 20 mg. of acetone-ether-treated mitochondria, experiments were carried out

in which choline dehydrogenase activities of the supernatant layer and residue were followed for various levels of sodium choleate added. The results of these experiments are presented in Fig. 3. Here it can be seen that, with increasing choleate concentration, the activity of the supernatant solution rapidly increased with a reciprocal fall in the activity of the residue. A broad maximum extending from 3.6 to 12 mg. of sodium choleate per 20 mg. of dried mitochondria per ml. of buffer was obtained. Beyond that point the choleate inhibited the choline dehydrogenase ac-

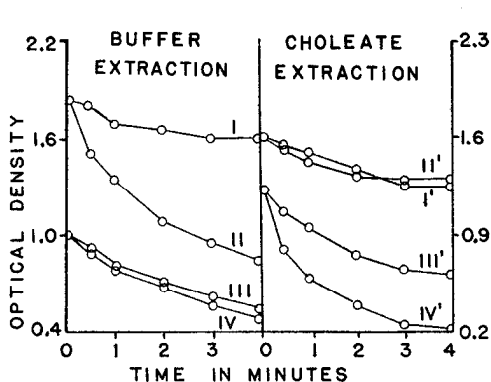


FIG. 2

Fig. 2. Experiments showing the extraction of choline dehydrogenase from acetone-dried mitochondria with buffer plus choleate but not with buffer alone. Curve I, residue control; Curve II, residue + choline; Curve III, soluble extract control; and Curve IV, soluble extract + choline.

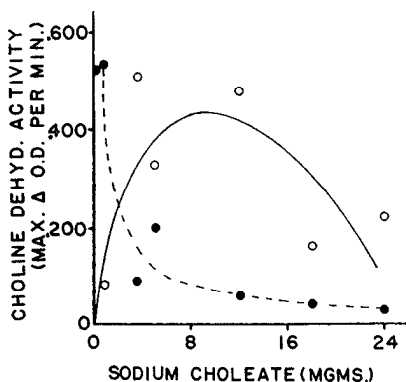


FIG. 3

Fig. 3. Relationship between the choline dehydrogenase activity remaining in the mitochondrial residue and passing into the soluble extract to the level of sodium choleate used for extraction. The solid curve denotes activity of the choleate extract. The broken curve denotes activity of the residue remaining after choleate extraction. See the text for the method used for calculating these results.

tivity. In succeeding experiments 3.6 mg. of sodium choleate were routinely employed for extracting 20 mg. of acetone-ether-treated mitochondria per ml. of buffer.

By using freshly isolated mitochondria which had not been treated with acetone and ether, it was found that extraction with sodium choleate also brought the choline dehydrogenase into solution. However, the mitochondria cannot be stored in the wet condition because of loss in activity (5). Therefore, since the acetone-dried mitochondria can be stored without loss in activity, it is more convenient to prepare a supply and use it as needed.

One of the limiting factors in the spectrophotometric assay method is the presence of fairly high endogenous activity in certain dried mito-

chondrial preparations. Under those conditions the concentration of the 2,6-dichlorophenol indophenol becomes limiting with respect to choline dehydrogenase activity. If higher dye concentrations are used to attempt to overcome this effect, the zero time optical density reading is too high (>2.0). Most of the endogenous dye reduction, however, can be removed by suspending the dried mitochondria first in 0.1 M sodium phosphate buffer (pH 6.8), centrifuging, and then extracting the mitochondrial residue with the sodium choleate-buffer mixture. Apparently this endogenous activity is due to oxidizable metabolites that are not completely removed during the original isolation procedure but which can be removed by washing the dried mitochondria with buffer before extracting with choleate.

TABLE I
Specific Activity of Choline Dehydrogenase (Based on Nitrogen) of Various Preparations of Mitochondria

Enzyme preparation	Specific choline dehydrogenase activity as maximal changes in optical density per min. per mg. N* $\times 1000$
Fresh mitochondria.....	290
Choleate extract of fresh mitochondria.....	685
Acetone-dried mitochondria.....	180
" " washed with buffer.....	420
Choleate extract of acetone-dried mitochondria.....	2400

* See the text for the method of calculation.

In order to follow the degree of purification of choline dehydrogenase from the freshly isolated mitochondria to the soluble extract containing the enzyme, the activity was determined at each step and calculated in terms of nitrogen present. The nitrogen of each fraction was measured with a micro-Kjeldahl procedure (Table I). The extraction of fresh, undried mitochondria with buffer and choleate more than doubled the specific activity based on nitrogen. The whole acetone-dried mitochondria possessed slightly less specific activity than fresh mitochondria, which may be due to enzyme inactivation during the drying process. When the dried mitochondria were washed with buffer before measuring the activity, the specific activity was more than twice that of the unwashed dried mitochondria. Preparation of soluble choline dehydrogenase from the acetone-dried mitochondria gave a 13-fold greater specific activity than that of the whole, dried mitochondria. Therefore, it appears that treatment of the dried mitochondria with choleate extracts the choline dehydrogenase but leaves most of the other protein of the dried mitochondria in the residue.

Extraction of Other Enzymes from Mitochondria by Sodium Choleate—

Two other enzymes known to be located in mitochondria, succinic dehydrogenase (15) and betaine aldehyde dehydrogenase (16), were assayed in the choleate extract prepared from acetone-dried mitochondria. By using 0.2 ml. of 0.15 M sodium succinate and 0.2 ml. of 2 per cent betaine aldehyde (17) as substrate with the other cuvette components, the same as for the choline dehydrogenase assay, it was found that both succinic and betaine aldehyde dehydrogenases were extracted from the mitochondria. In quantitative terms, the activity for succinic dehydrogenase was 120 units of change in optical density \times 1000 per minute and for betaine aldehyde oxidase, 60 units of change in optical density \times 1000 per minute.

To observe whether the complete choline and succinic acid oxidase systems were extracted by choleate, the soluble mitochondrial extracts were studied manometrically with oxygen uptake as the criterion for the presence of the complete dehydrogenase-cytochrome system. Since no aerobic activity could be detected for either enzyme (with or without added cytochrome *c*), it can be concluded that at least one necessary component was not extractable by choleate. When the whole acetone-dried mitochondria from which the soluble extracts were made were tested for choline oxidase activity, they were found to utilize oxygen as the hydrogen acceptor. This indicates that, while the complete oxidative system is present in the acetone-dried mitochondria, only certain of the enzyme components of the complete system are extracted by choleate.

SUMMARY

1. Choline dehydrogenase has been extracted from rat liver mitochondria with sodium choleate and phosphate buffer as the dispersing system.
2. The soluble enzyme utilizes 2,6-dichlorophenol indophenol as a hydrogen acceptor but not molecular oxygen, indicating that all of the mitochondrial components of the choline oxidase system are not extracted by choleate.
3. By the extraction procedure, a 13-fold purification of choline dehydrogenase has been obtained.
4. Among other enzymes tested, mitochondrial succinic and betaine aldehyde dehydrogenases were also brought into solution by choleate extraction.

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