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CHOLINE AND BETAINE ALDEHYDE OXIDATION BY RAT LIVER MITOCHONDRIA

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SUMMARY

1. The question of the ability or inability of rat liver mitochondria to oxidize externally added or internally generated betaine aldehyde has been reexamined. Well washed mitochondria were demonstrated to contain approx. 7% of the post-nuclear betaine aldehyde dehydrogenase as an integral component. The enzyme is approximately equally distributed between the inner membrane and the inter-membrane *plus* matrix fractions. Significantly, none was found in the outer membrane fraction. The mitochondrial enzyme was shown to be functional under all the conditions tested; betaine aldehyde generated within the mitochondria by choline oxidation or added externally was oxidized to betaine in significant amounts.

2. The stoichiometry for the complete oxidation of choline or externally added betaine aldehyde was confirmed to be 2 and 1 moles, respectively, of O_2 utilized per mole of substrate added. Depending on the reaction conditions employed, considerable variation in the relative amount of choline oxidase and betaine aldehyde oxidase activities of mitochondria was observed when they were allowed to oxidize only a portion of the choline added. The necessity of measuring the contribution of betaine aldehyde oxidase in studies of choline oxidase is discussed.

3. Reasons for the discrepancies in the literature concerning the ability of mitochondria to oxidize betaine aldehyde are discussed.

INTRODUCTION

MANN AND QUASTEL[1] demonstrated that rat liver preparations oxidized choline to betaine aldehyde. Additional studies[2,3] demonstrated that under the appropriate conditions, betaine aldehyde was further oxidized to betaine. The subcellular site of choline oxidation has since been shown to be the mitochondrion[4,5].

The subcellular site of betaine aldehyde oxidation is less clear. It is known that the cytosol contains betaine aldehyde dehydrogenase (betaine aldehyde:NAD oxidoreductase, EC 1.2.1.8) activity[6,7]. On the other hand, there are conflicting reports in the literature about the ability of mitochondria to oxidize either externally added betaine aldehyde or betaine aldehyde presumably generated within them during choline oxidation. In contrast to some reports[8,9], others[10,11] have indicated

that mitochondria are able to oxidize externally added betaine aldehyde. Studies on the complete oxidation of choline by rat liver mitochondria indicated that 2 atoms of oxygen were required per mole of choline added[12,13]. These results are consistent with the interpretation that betaine aldehyde formed within mitochondria as a result of choline oxidation may be further oxidized to betaine. In studies of mitochondrial choline oxidation in which the substrate is incompletely utilized, rarely have the products been directly characterized to ascertain how much, if any, of the betaine aldehyde formed was further oxidized to betaine. When this has been done, different results have been reported by different investigators. For example, as little as 0 (ref. 9) to 7 % (ref. 14) or as much as 84 % (ref. 11) of the choline oxidized by mitochondria could be isolated as betaine reineckate. Attempts to detect betaine aldehyde dehydrogenase activity in rat liver mitochondria also have been contradictory. It has been reported to have been present in extracts of mitochondrial acetone powders[15] or extracts of freshly prepared mitochondria[11,12]. However, it has also been reported to be absent from sonic extracts of mitochondria[9].

The considerable discrepancy in the literature concerning the ability or inability of rat liver mitochondria to oxidize betaine aldehyde has prompted us to reinvestigate this question. It will be unequivocally shown that mitochondria do have the ability to oxidize either externally added or internally generated (from choline) betaine aldehyde to betaine. This oxidation is due to betaine aldehyde dehydrogenase activity within the mitochondria as opposed to cytoplasmic betaine aldehyde dehydrogenase activity adsorbed to the surface of mitochondria.

METHODS

General

Preparation of mitochondria, manometric respiration measurements, betaine aldehyde assays, protein assays and estimation of choline oxidase activities from O_2 consumption and betaine aldehyde formation were normally conducted as previously described[16], except that reactions were terminated by 2.4 % $HClO_4$ (final concn.) rather than by trichloroacetic acid. All manometric assays were performed at 30°. Each manometric assay contained 17 mM Tris–HCl buffer (pH 7.5), 10 mM potassium phosphate buffer (pH 7.5), 5 mM $MgSO_4$, and 117 mM mannitol. This "basic mixture" was supplemented with other components as indicated in the appropriate figure or table. After mitochondria were separated from homogenates, the cytosol was separated from the microsomes by centrifugation at 105000 × *g* for 1 h.

Qualitative and quantitative estimation of reaction products

Products of mitochondrial [^{14}C]choline oxidation in KOH-neutralized supernatant solutions from reactions terminated with $HClO_4$ were separated from one another by paper chromatography in *n*-butanol–ethanol–acetic acid–water (8:2:1:3, by vol.)[17] or electrophoresis at pH 6.5 in pyridine–acetic acid–water (300:12:2700, by vol.)[18] for 25 min at 3000 V. Unlabeled standards or products were located with Dragendorff's reagent[19]. ^{14}C-labeled reactants and products were located by autoradiography. Radioactive areas were eluted with water and counted in Bray's scintillation fluid[20] using a Tricarb liquid-scintillation spectrometer. The counting data were used to calculate the amount of each product as well as the relative

contribution of the choline oxidase and betaine aldehyde oxidase activities to the total oxidase activity of mitochondria metabolizing choline. The per cent betaine aldehyde oxidase activity was calculated* as follows: 100 × (μmoles betaine)/(μmoles betaine aldehyde + 2 × μmoles betaine).

Identification of the product of mitochondrial oxidation of unlabeled betaine aldehyde was done by a scaled-down modification of the procedure of CHRISTIANSON *et al.*[21] as follows: The reaction mixture (KOH-neutralized supernatant solution) was made 1 M in HCl and applied to a 0.9 cm × 55 cm column of Biorad AG 50W-X8 H^+ form resin after the resin had been washed with 100 ml of 1.5 M HCl. The column was washed with 100 ml of 1.5 M HCl followed by 100 ml of 2.5 M HCl. The betaine or betaine aldehyde was present in the 2.5 M HCl eluate. Betaine was detected[22] directly in aliquots of the 2.5 M HCl eluate and betaine aldehyde was detected[16] after evaporation of the 2.5 M HCl eluate to dryness *in vacuo*. The remainder of the fractions containing betaine or betaine aldehyde was concentrated to dryness *in vacuo*. The identity of the compound in the concentrate was verified by paper electrophoresis as described above.

Preparation of submitochondrial fractions

In the experiments on the submitochondrial location of betaine aldehyde dehydrogenase, the conditions for the preparation of subcellular and submitochondrial fractions were as described by SOTTOCASA *et al.*[23] with the possible exception of the sonic oscillation conditions. The swollen-recontracted mitochondria were submitted to sonification in 3.5-ml volumes for 15 sec each using a Branson Model W-185-C sonifier fitted with a "Standard" 6.76-inch micro tip. The settings were placed at the minimum power output (Setting 1) attained by the instrument. This yielded a meter reading of about 85 during sonification. The sonicate was separated into "heavy, light and soluble" fractions by centrifugation using their[23] three-layer sucrose density gradient system. Only in this particular experiment was protein assayed by the method of LOWRY *et al.*[24].

Enzyme assays

All enzyme assays listed below were conducted at 25° except for glucose-6-phosphatase which was done at 37°. Betaine aldehyde dehydrogenase was assayed under conditions similar to those previously described[7]. The reaction mixtures contained 100 mM Tris–HCl buffer (pH 9), 1 mM NAD^+, 10 mM NaOH-neutralized cysteine·HCl, 5 mM $MgCl_2$, enzyme and either 0.2 or 1 mM betaine aldehyde in a final volume of 1 ml. The reaction was initiated by the addition of betaine aldehyde. Anaerobic assays for betaine aldehyde dehydrogenase were performed as above except that the reaction mixture was scaled up 3-fold, and conducted in Thunberg cuvettes under a N_2 atmosphere. The reagents were preflushed with O_2-free N_2 (ref. 25). NADH oxidase assays were conducted under the following conditions: 100 mM Tris–HCl buffer (pH 9), 5 mM $MgCl_2$, 0.1 mM NADH in a volume of 1 ml. Succinate–cytochrome *c* reductase and rotenone-insensitive cytochrome *c* reductase assays were

* The calculation is based on the fact that the total oxidase activity observed during mitochondrial choline oxidation is the sum of the betaine aldehyde formed and 2 times the betaine formed since two oxidative steps are required for the formation of betaine from choline. Betaine aldehyde oxidase activity is equivalent to the betaine formed.

performed according to SOTTOCASA *et al.*[26]. Glucose-6-phosphatase was assayed according to SWANSON[27]. Adenylate kinase was assayed as described by SOTTOCASA *et al.*[23] except for the following modifications. The ADP concentration was decreased from 5 to 0.5 mM and only 2 units of hexokinase and 2 units of glucose-6-phosphate dehydrogenase were present in the reaction mixture. A unit of enzyme activity is the formation of 1 μmole of product per min under the assay conditions used. Specific activity is the units/mg protein.

MATERIALS

The betaine aldehyde used was either isolated from an incubation mixture containing choline and rat liver mitochondria as described by JELLINEK *et al.*[14] or was a gift from Dr. M. Jellinek of the St. Louis University School of Medicine. Unlabeled choline was obtained from Nutritional Biochemicals Co. NAD^+, NADH, ATP, ADP, uniformly-labeled [^{14}C]betaine and [1,2-$^{14}C_2$]choline were obtained from Calbiochem. Glucose 6-phosphate, cytochrome *c*, rotenone and glucose-6-phosphate dehydrogenase were obtained from Sigma. Crystalline hexokinase was obtained from Boheringer. Sigma Type III hexokinase was a gift from Dr. D. S. Goldman of the Veterans Administration Hospital, Madison, Wisc. All other chemicals used were of reagent quality.

RESULTS

Mitochondrial betaine aldehyde dehydrogenase

We have reinvestigated whether or not rat liver mitochondria contain betaine aldehyde dehydrogenase since claims of its presence[11,12,15] and its absence[9] have been reported. Fig. 1 shows the betaine aldehyde dehydrogenase activity of the fractions

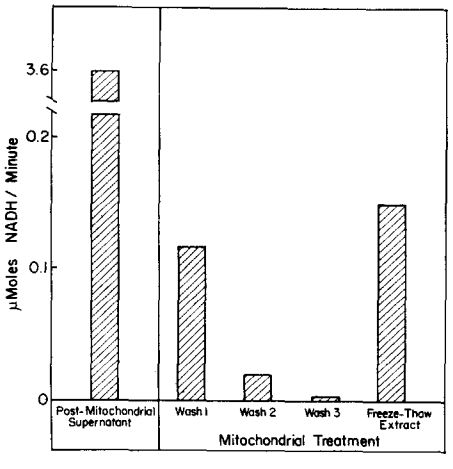

Fig. 1. Betaine aldehyde dehydrogenase activity at various stages during the preparation of mitochondrial freeze–thaw extracts. Mitochondria were prepared (METHODS) and then washed 3 times with 0.25 M sucrose. The mitochondrial freeze–thaw extract was prepared from the mitochondrial pellet obtained after the third sucrose wash as described in the text. Supernatant solutions obtained after washing the mitochondria or preparing the freeze–thaw extract were assayed for betaine aldehyde dehydrogenase and NADH dehydrogenase activities. The latter, which would interfere with betaine aldehyde dehydrogenase, was essentially absent. The total enzyme activity of each treatment is shown on a per g wet wt. of liver basis.

examined during the preparation of mitochondrial freeze–thaw extracts. The post-mitochondrial supernatant solution contained the majority of the post-nuclear betaine aldehyde dehydrogenase activity. This is in agreement with previous observations. Essentially none of this activity was in the microsomal fraction, but was found nearly exclusively in the cytosol[6,7]. Essentially all of the betaine aldehyde dehydrogenase which could be washed from the mitochondria with cold sucrose was removed by washing them 3 times. After the third sucrose wash, the mitochondria (suspended in 0.25 M sucrose) were subjected to freezing (−80°) and thawing (25°) for six complete cycles, centrifuged, and the supernatant solution was assayed. The activity of the freeze–thaw extract was higher than even the first mitochondrial sucrose wash. Thus, disruption of the well washed mitochondria by freezing and thawing them liberated betaine aldehyde dehydrogenase activity. The amount extracted was 3.8% of the total activity of the fractions shown in Fig. 1.

In experiments not shown, mitochondrial sonicates, which were previously said to be devoid of betaine aldehyde dehydrogenase activity[9], were examined for this activity. A spectrophotometric assay (METHODS) was used in which NADH formation is monitored. Betaine aldehyde dehydrogenase activity was readily detectable, but only after a lag period of about 5–20 min. The reason for this lag period resulted from the high levels of NADH dehydrogenase (oxidase) activity in mitochondrial sonicates. Thus, the formation of NADH was masked until the cuvette became anaerobic. This conclusion was verified by conducting betaine aldehyde dehydrogenase assays under anaerobic conditions which resulted in the elimination of the lag period.

Higher betaine aldehyde dehydrogenase activities were generally observed in mitochondrial sonicates than in freeze–thaw extracts. As much as 7% of the post-nuclear betaine aldehyde dehydrogenase activity was found in mitochondrial sonicates.

Submitochondrial location of betaine aldehyde dehydrogenase

SOTTOCASA *et al.*[23] reported a procedure for the preparation of three subfractions of mitochondria based on mitochondrial swelling and recontraction, followed by sonic oscillation and discontinuous gradient centrifugation. These mitochondrial subfractions were believed to be derived from the inner membrane *plus* some matrix material (heavy fraction), outer membrane (light fraction) and matrix *plus* intermembrane materials (soluble fraction). Several enzymes were found to be present predominantly in one or another of the three subfractions[23,26]. Rat liver mitochondria were submitted to this procedure and all post-nuclear fractions obtained were assayed for betaine aldehyde dehydrogenase in an attempt to determine its ease of release from mitochondria under conditions of mitochondrial swelling (Table I) and to determine its submitochondrial location (Table II).

The results shown in Table I demonstrate that of the total betaine aldehyde dehydrogenase which could be released by sonification of the mitochondria, almost none was released either by washing with isotonic sucrose or by swelling the mitochondria in dilute salt solution (Tris–phosphate buffer). The total betaine aldehyde dehydrogenase released by these two treatments, *plus* the further treatment of recontraction of the mitochondria, liberated no more than 10% of the total mitochondrial enzyme released by sonification. These results strongly suggest that the

betaine aldehyde dehydrogenase activity associated with the mitochondrial fraction (6% of the total post-nuclear activity in this experiment) is not simply cytoplasmic enzyme weakly adsorbed to the mitochondria.

Table II shows the submitochondrial distribution of betaine aldehyde dehydrogenase. To be able to assess the quality of the three mitochondrial subfractions we obtained, compared to those obtained by SOTTOCASA *et al.*[23], adenylate kinase, rotenone-insensitive NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase activities of the three subfractions were also measured. These enzyme activities were previously shown to reside primarily in the soluble, light, and heavy fractions, respectively. In general, the submitochondrial distribution of the marker enzymes was in good qualitative agreement with those of SOTTOCASA *et al.*[23]. The relative amount of protein in the three subfractions was somewhat different from that reported by them. We may have used somewhat stronger sonification conditions which resulted in the liberation of more protein from the heavy into the soluble fraction than they observed. Nearly equal betaine aldehyde dehydrogenase activity was found in the heavy (inner membrane *plus* some matrix) and soluble (matrix *plus* intermembrane material) fractions while no activity was found in the light (outer membrane) fraction. Thus, that portion of the cellular betaine aldehyde dehydrogenase which is mitochondrial has a submitochondrial distribution which resembles that of other mitochondrial NAD^+-linked dehydrogenases[23]. Due to the difference in protein distribution described above, the specific activity of the heavy fraction was higher

TABLE I

LACK OF SIGNIFICANT RELEASE OF BETAINE ALDEHYDE DEHYDROGENASE FROM SWOLLEN OR RECONTRACTED MITOCHONDRIA

Assays were conducted as described in METHODS. Only the whole mitochondrial sonicate was assayed for betaine aldehyde dehydrogenase under anaerobic conditions. NADH dehydrogenase activity of the other three fractions demonstrates that the aerobic betaine aldehyde dehydrogenase assays could not have been greatly underestimated due to NADH dehydrogenase activity of the fraction. After mitochondria were collected they were washed twice with sucrose. The mitochondrial pellet from 2 g liver (41 mg protein) was suspended in Tris–phosphate buffer 5 min at 0° to allow the mitochondria to swell. Sucrose, ATP and $MgSO_4$ were added to give the final concentration shown and the mitochondria were allowed to recontract for 5 min at 0° prior to submitting the mixture to sonic oscillation (METHODS). At the end of the periods during which the mitochondria were allowed to swell or recontract, aliquots were removed and centrifuged 10 min at 20000 × *g*. These supernatant solutions and the sucrose wash were assayed as indicated. The whole mitochondrial sonicate was assayed without prior centrifugation. More detailed instructions for Procedures 1–4 are found in ref. 23.

Fraction assayed	*Medium*	*Assay (munits/g liver)*	
		Betaine aldehyde dehydrogenase	*NADH dehydrogenase*
(1) 2nd mitochondria wash	0.25 M sucrose	0	7
(2) Swollen mitochondria supernatant solution	10 mM Tris–phosphate buffer (pH 7.5)	2	0
(3) Swollen–recontracted mitochondria supernatant solution	0.45 M sucrose, 0.5 M ATP, 0.5 mM $MgSO_4$, 7.5 mM Tris–phosphate buffer (pH 7.5)	12	2
(4) Whole mitochondria sonicate	Same as 3	148	Not assayed

TABLE II

SUBMITOCHONDRIAL DISTRIBUTION OF BETAINE ALDEHYDE DEHYDROGENASE AND MARKER ENZYMES

Assays were performed as described in METHODS. All enzyme assays were linear with respect to amount of fraction assayed. Anaerobic conditions were used for the betaine aldehyde dehydrogenase assays to avoid interference by NADH oxidase activity. H, L and S refer to heavy, light and soluble submitochondrial subfractions, respectively. Percent activity means the ratio of the activity in the fraction tested to the sum of the activities of the three fractions × 100. The relative specific activity is the ratio of the specific activity of the submitochondrial fraction compared to the specific activity of the whole mitochondrial sonicate. Percent recovery means the sum of the activities of the three subfractions compared to the whole sonicate × 100. In the whole sonicate 100% activity for each enzyme was as follows: (enzyme, munits/mg protein); adenylate kinase, 595; rotenone-insensitive NADH–cytochrome *c* reductase, 332; succinate–cytochrome *c* reductase, 117; and betaine aldehyde dehydrogenase, 7.3. 20.3 mg of mitochondrial protein per g wet wt. liver were present in the whole mitochondrial sonicate. The mitochondria used in this experiment contained 7.4% of the post-nuclear glucose-6-phosphatase activity, thus indicating they were contaminated to a small extent by microsomes.

	Assay														
	Adenylate kinase			*Rotenone-insensitive NADH–cytochrome c reductase*			*Succinate–cytochrome c reductase*			*Betaine aldehyde dehydrogenase*			*Protein*		
	H	*L*	*S*	*H*	*L*	*S*	*H*	*L*	*S*	*H*	*L*	*S*	*H*	*L*	*S*
Activity (%)	Trace	5	95	7	62	31	97	3	Trace	55	0	45	39	15	45
Relative specific activity	—	0.3	2.6	0.2	3.7	0.6	2.1	0.1	—	1.9	—	1.3	—	—	—
Recovery (%)		106			81			78			115			86	

than that of the soluble fraction in contrast to observations[23] with other NAD^+-linked dehydrogenases.

Since no betaine aldehyde dehydrogenase activity was observed in the outer membrane fraction while about 50% was found in the inner membrane fraction, it is concluded that the betaine aldehyde dehydrogenase activity associated with the mitochondria is truly mitochondrial and not simply due to tightly adsorbed cytoplasmic enzyme.

Qualitative and quantitative identification of the products of both "complete" and "incomplete" oxidation of choline by mitochondria

Previously it was reported that the rate of mitochondrial choline oxidation was markedly influenced by conditions[16] which either maintained or allowed a relaxation of a mitochondrial permeability barrier toward choline[28]. When the mitochondrial permeability barrier was functioning, the rate of "choline" oxidation was slow. The rate was increased 3–5-fold under conditions in which the permeability barrier was relaxed. Since the amount of betaine aldehyde produced was always less than the O_2 consumed, it was assumed that some of the betaine aldehyde had been further oxidized to betaine. Based on this assumption it was concluded that when the choline permeability barrier was functioning, much (up to 40%) of the O_2 utilization was due to betaine aldehyde oxidation, but that when the permeability barrier was relaxed, relatively little (about 15%) of the oxidation was due to betaine aldehyde

oxidation. This assumption and conclusions were challenged when it was reported that mitochondria are unable to oxidize betaine aldehyde[9].

Choline oxidation has been reexamined using ^{14}C-labeled choline as substrate. O_2 utilization and ^{14}C-labeled products were measured. Assays were performed under a variety of conditions to attempt to find out if betaine is produced under some conditions but not others. When [^{14}C]choline was oxidized until O_2 consumption had ceased (complete oxidation) it was found that 1.8 atoms of oxygen had been utilized per mole of choline added to the reaction mixture. Only one radioactive product, which had the mobility of betaine, could be detected by paper chromatography and autoradiography. Fig. 2 and Table III present qualitative and quantitative results, respectively, of experiments in which only part of the added choline was allowed to be oxidized under conditions of either a functioning (Lane 4, Fig. 2; Expt. 1, Table III) or a relaxed (Lane 5, Fig. 2; Expt. 2, Table III) choline permeability barrier. When only a small part of the added choline is oxidized (15 % Expt. 1, Table III) under conditions of a functioning permeability barrier, the major oxidation product is betaine. When the permeability barrier is relaxed, considerably more choline is oxidized (50 %, Expts. 2 and 3, Table III). Although a significant amount

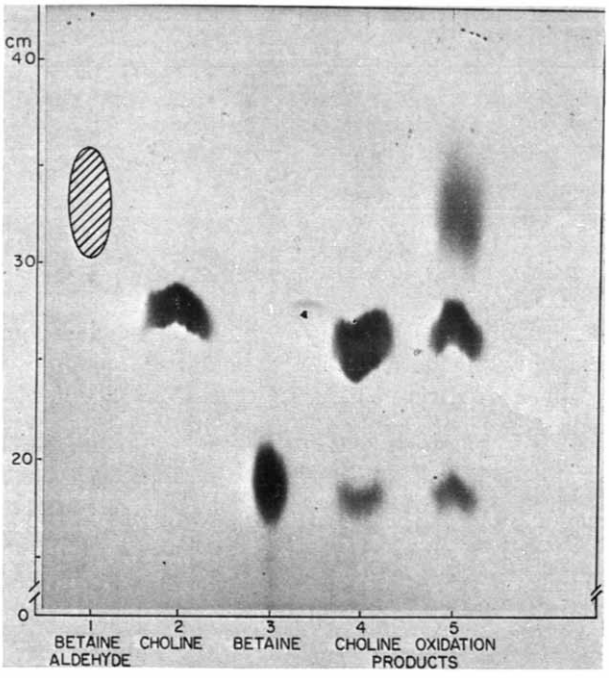

Fig. 2. Chromatographic identification of the products of the incomplete oxidation of choline by rat liver mitochondria. The samples in the lanes were: Lane 1, betaine aldehyde; Lane 2, [^{14}C]-choline; Lane 3, [^{14}C]betaine; Lanes 4 and 5, mitochondrial [^{14}C]choline oxidation products. The conditions of incubation for the formation of the choline oxidation products included the basic mixture (METHODS) supplemented as indicated. Lane 4, 13 mM KF, 1 mM ATP, 8.9 mg mitochondria (as protein), 0.5 mg Sigma Type III hexokinase, and 10 mM [^{14}C]choline (56.7 nC per μmole). Under these conditions the choline permeability barrier is operative[16]. Lane 5, same as Lane 4 except that ATP and KF were omitted (relaxed permeability barrier conditions[16]). The mixtures were incubated 90 min. Chromatography, autoradiography and detection of betaine aldehyde were conducted as described in METHODS.

of betaine also is formed under these conditions, the oxidation of betaine aldehyde is not increased to nearly the same degree as is the oxidation of choline. Thus, betaine aldehyde accumulates as the major product. It is concluded that under a variety of conditions a significant amount of the betaine aldehyde formed within the mitochondrion by choline oxidation is further oxidized to betaine.

TABLE III

RELATIVE CONTRIBUTION OF CHOLINE AND BETAINE ALDEHYDE OXIDASE* ACTIVITIES OF RAT LIVER MITOCHONDRIA OXIDIZING EXTERNALLY ADDED [^{14}C]CHOLINE

[^{14}C]Choline was oxidized by rat liver mitochondria under the following conditions: Expt. 1, same as described for Lane 4, Fig. 2. The choline and hexokinase were added to the main compartment from the side arm of the Warburg flasks after a temperature equilibration period of 10 min. The reaction was terminated after 90 min. Expt. 2 was the same as described for Lane 5, Fig. 2. Expt. 3 was the same as Expt. 2 except that 50 μg of crystalline hexokinase was substituted for Type III hexokinase. (The amounts of the two preparations of hexokinase used were equivalent on an activity basis.) O_2 uptake was corrected by the change in gas tension observed in reaction mixtures to which no choline was added. Chemical refers to information obtained or calculated[16] from the manometric measurement of O_2 and chemical determination of betaine aldehyde[16]. ^{14}C refers to information obtained or calculated (METHODS and ref. 29) by the radioisotope assay. See METHODS for details of assay procedures.

Expt.	*Oxygen utilized* (μ*atoms*) *Chemical*	*Betaine aldehyde found* (μ*moles*)		*Betaine found* (μ*moles*) ^{14}*C*	*Choline oxidase* (%)		*Betaine aldehyde oxidase* (%)	
		Chemical	^{14}*C*		*Chemical*	^{14}*C*	*Chemical*	^{14}*C*
1	4.7	0.4	1.3	3.6	54.5	57.7	45.5	42.3
	6.9	0.2	1.3	3.2	51.5	58.4	48.5	41.6
2	20.0	12.4	12.0	4.3	81.0	79.1	19.0	20.9
	15.1	9.7	10.9	3.5	82.0	80.4	18.0	19.6
3	20.4	13.6	12.4	4.0	83.5	80.4	16.5	19.6

In the experiments shown in Table III two of three recently discussed[29] independent methods for measuring both the choline and betaine aldehyde oxidase* activities of mitochondria were employed. One of the procedures is based on the manometric measurement of O_2 utilization and chemical determination of betaine aldehyde formation. The use[16] of this method has been criticized[9] because it requires the assumption that betaine aldehyde is oxidized by mitochondria and because crude preparations of hexokinase used were thought[9] to interfere. The close agreement of the results of this indirect assay with those of the direct assay, which employs ^{14}C-labeled choline, as well as the agreement between Expts. 2 and 3, in which crude and crystalline hexokinase, respectively, were used, demonstrates the adequacy of the former assay. The results also confirm and extend (by direct measurement of the products) previously drawn conclusions[16] discussed above which were based on the results of the less rigorous indirect assay. The results shown in Table III clearly

* Of the assays described in Table III, the manometric assay measures the oxidase activities, while the ^{14}C assay measures the dehydrogenase activities for choline and betaine aldehyde. Since the results of the two assay methods are essentially the same, for simplicity, the term oxidase will be used in this section of the manuscript and Table III to apply to oxidase and dehydrogenase activities.

demonstrate that the relative activities of the two oxidase activities may vary considerably depending on the conditions employed. Therefore, it should be stressed that measurement of O_2 utilization alone is an insufficient assay to accurately test the effects of experimental manipulation on choline oxidase, as sometimes has been done in the past, since such manipulations could affect the betaine aldehyde oxidase as well as, or instead of, choline oxidase.

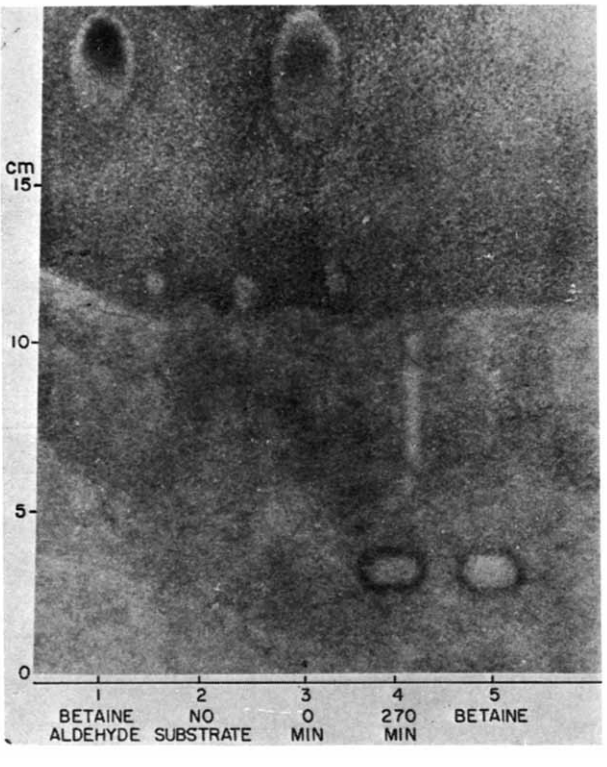

Fig. 3. Identification of betaine as the product of mitochondrial oxidation of betaine aldehyde. Electrophoresis (METHODS) of the following samples was conducted for 25 min: Lanes 1 and 5 were betaine aldehyde and betaine, respectively. Aliquots of reaction mixtures described in the text, with the indicated modifications, were purified prior to electrophoresis as described in the METHODS. Lane 2, incubated 270 min but contained no substrate; Lane 3, contained betaine aldehyde but the reaction was stopped at zero time by acidification; Lane 4, reaction mixture described in the text after a 270-min incubation.

Stoichiometry and identification of the product of mitochondrial choline oxidation of externally added betaine aldehyde

10 μmoles of betaine aldehyde were incubated in the manometric assay basic mixture (METHODS) supplemented with 13 mM glucose, 0.33 mM ADP, 50 μg crystalline hexokinase and 5.2 mg (as protein) of mitochondria until O_2 uptake ceased (270 min). At this time 9.2 μatoms of oxygen had been utilized and spectrophotometric assay[16] indicated that 9.8 μmoles of betaine aldehyde had disappeared. Thus, the expected stoichiometry of 1 atom of oxygen per mole of betaine aldehyde oxidized was observed. After purification by column chromatography (METHODS) the identity

of the product was established by paper electrophoresis. Fig. 3 shows that during the 270-min incubation all of the betaine aldehyde was converted to betaine. These results show that under the conditions employed, mitochondria can oxidize externally added as well as internally generated betaine aldehyde.

DISCUSSION

During reexamination of the question of the ability or inability of rat liver mitochondria to oxidize betaine aldehyde, it was routinely observed that 4–7 % of the post-nuclear betaine aldehyde dehydrogenase activity was associated with the mitochondrial fraction. The remainder was present in the cytosol. It was therefore necessary to demonstrate that the mitochondrial activity was not due to cytoplasmic enzyme adsorbed to the surface of the mitochondria during their preparation. Several attempts were made to distinguish cytoplasmic enzyme from mitochondrial enzyme. Much of the cytoplasmic enzyme activity which was largely inactivated (70 %) during chromatography on DEAE-cellulose could be restored by a time- and temperature-dependent incubation with NAD^+ and cysteine at pH 9. Nearly all (97 %) of the mitochondrial activity was lost during chromatography under the same conditions, but in contrast to cytoplasmic enzyme essentially none of it could be reactivated. These results suggested that the cytoplasmic and mitochondrial enzyme might be different. While they appeared to be partially separable by DEAE-cellulose chromatography, but not Sephadex-200 chromatography or electrofocusing (isoelectric point near pH 6), the separation was not entirely convincing, partly because of large losses in activity. Therefore, an indirect, nevertheless convincing, demonstration that the mitochondrial activity was not simply due to cytoplasmic enzyme adsorbed to the surface of the mitochondria was employed. Had cytoplasmic enzyme been weakly adsorbed to the mitochondria, it should have been readily washed from them by an isotonic nonionic solution (0.25 M sucrose) or hypotonic ionic solution (0.01 M Tris–phosphate buffer) both of which were employed. These treatments consistently failed to remove the betaine aldehyde dehydrogenase activity from the mitochondria. Thus, either the mitochondrial activity must have been an integral part of the mitochondria or else cytoplasmic enzyme was tightly bound to them. Were the latter possibility correct, one might then expect part or all of any adsorbed activity to remain bound to the outer mitochondrial membrane during fractionation of the mitochondria into submitochondrial components. However, essentially none of the mitochondrial activity was associated with the outer membrane-rich fraction of the subfractionated mitochondria but was found with the fractions corresponding to the matrix, inner membrane and intramembrane fractions. Thus, it is concluded that betaine aldehyde dehydrogenase is an integral component of rat liver mitochondria.

The occurrence of betaine aldehyde dehydrogenase in mitochondria appeared to be inconsistent with reports that rat-liver mitochondria have little[14,30] or no ability[8,9] to oxidize betaine aldehyde generated during choline oxidation and no ability to oxidize externally added betaine aldehyde[9]. Therefore, it seemed desirable to carefully examine the nature of the product(s) of choline oxidation under a variety of conditions to ascertain whether or not the mitochondrial betaine aldehyde dehydrogenase is functional under some conditions but not others. Under the three types of

conditions of choline oxidation tested, betaine was always formed. Thus, the betaine aldehyde dehydrogenase was functional.

Externally added betaine aldehyde was also oxidized to betaine under the conditions used. In experiments not described, externally added betaine aldehyde was incubated with mitochondria under the conditions of YUE *et al.*[9] which included fluoride ion. Only a slow oxidation was observed. In the absence of F^- similar results were obtained during the first 30–40 min of incubation, however, the rate of oxidation then nearly quadrupled. Somewhat similar observations have been reported with regard to choline oxidation[16] and it was concluded that the F^- indirectly led to the maintenance of a permeability barrier to choline. The effect of F^- on betaine aldehyde oxidation appears to be limited to externally added betaine aldehyde since results presented in Table III (Expts. 1 and 2) show that the oxidation of internally generated betaine aldehyde is not greatly different in the presence or absence of F^-. These results suggest that the mitochondria may also exhibit a permeability barrier to betaine aldehyde. This possibility has not been directly tested as it has for choline[28]. The mitochondrion is the major site of betaine aldehyde production from choline. Thus, if the mitochondria exhibit a permeability barrier to betaine aldehyde *in vivo*, and if this barrier affects the outward as well as inward flux of betaine aldehyde, then the mitochondrion might be the major site of betaine aldehyde oxidation even though they contain only a small percentage of total liver betaine aldehyde dehydrogenase activity. This possibility remains to be tested experimentally.

It is concluded that rat liver mitochondria do contain significant amounts of functional betaine aldehyde dehydrogenase activity, and that the reasons it was not detected in significant amounts by previous workers were due mainly to the experimental conditions they used*.

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* The effects of relatively high phosphate concentration and temperature used[14] have been described[16]. The effects of F^- on the oxidation of externally added betaine aldehyde and the difficulties encountered in assaying betaine aldehyde dehydrogenase in mitochondrial sonicates were described in this paper.

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