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CHOLINE DEHYDROGENASE

ASSAY, PROPERTIES AND INHIBITORS

DEAN R. HAUBRICH* and NANCY H. GERBER
Neuropsychopharmacology Section, Department of Pharmacology, Merck Institute for Therapeutic Research, West Point, PA 19486, U.S.A.

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Abstract—Although choline has proven useful in the treatment of neurological disorders, its duration of action is limited by its rapid rate of metabolism. In order to evaluate the role of choline dehydrogenase in regulation of choline metabolism, some of the properties of the enzyme were studied using a new radioisotopic assay that is more sensitive and specific than previous methods. Choline dehydrogenase activity was present predominantly in kidney and liver of all species examined. Rats, mice, toads, cats, dogs, monkeys and sheep had highest activities in liver, whereas humans, baboons, rabbits and guinea pigs had more enzyme in kidneys. Enzyme activity was found to increase with age, reaching a maximum concentration at 1 week in livers and 4 weeks in kidneys of mice. Choline dehydrogenase, partially purified from rat liver mitochondria, was optimally active at a pH between 7 and 9, at a temperature of 37°, and in the presence of phenazine methosulfate. The K_m for choline was similar for both the soluble (5.7 mM) and the particulate (5.3 mM) enzyme but the V_{max} was slightly higher for the soluble [77 nmoles/min · (mg protein)$^{-1}$] as compared to the particulate [34 nmoles/min · (mg protein)$^{-1}$] preparation. When choline oxidation was studied *in vivo*, the concentration of free choline in liver (60 μM) and the rate of oxidation of [^{3}H]-choline administered intravenously [0.29 nmoles/min · (mg protein)$^{-1}$] were found to be markedly less than the respective K_m and V_{max} values determined *in vitro*. Thus, choline dehydrogenase may not be saturated with choline under physiologic conditions, which could explain the rapid rate of metabolism of exogenously administered choline. The product of the reaction, betaine aldehyde, was a potent competitive inhibitor of choline dehydrogenase (K_i = 0.6 mM), whereas other products of choline metabolism were weaker inhibitors (K_i values > 10 mM). Dimethylaminoethanol (deanol; a cholinomimetic agent) and 2-amino-2-methylpropanol inhibited the enzyme *in vitro* with inhibitory constants of 1.8 and 1.4 mM, respectively, and caused an increase in concentration of choline in kidney and liver when administered to mice, which suggests that choline dehydrogenase is an important enzyme in regulation of the concentration of free choline in tissues under physiologic conditions.

There is a great deal of interest in the role of choline in regulation of cholinergic neuronal activity since the discovery that administration of large doses of choline causes an increase in the concentration of acetylcholine in peripheral [1] and central [2, 3] cholinergic neurons. For example, choline has been given to patients suffering from neurological disorders that are thought to be related to deficits in cholinergic neurons, and some improvement has been reported (see Ref. 4). Furthermore, choline administered to laboratory animals has been shown to stimulate cholinergic neuronal activity in the brain, possibly by mass-action-induced stimulation of acetylcholine synthesis and release [5]. In addition, choline exerts direct stimulatory effects on muscarinic cholinergic receptors [6] and this effect may contribute to some of its pharmacologic actions.

In previous studies of the regulation of choline metabolism, choline given intraarterially to guinea pigs was found to be rapidly oxidized to betaine [7]. This finding seemed to contradict the results of other investigators who had been unable to detect oxidation of choline *in vitro* in livers of this species [8]. Choline is oxidized to betaine in two steps; the first is catalyzed by choline dehydrogenase (EC 1.1.99.1) which converts choline to betaine aldehyde, and the second by betaine aldehyde dehydrogenase (EC 1.2.1.8) which oxidizes the aldehyde to betaine. Choline dehydrogenase was discovered by Mann and Quastel in 1937 [9] and has been studied most extensively in rat liver. Although choline dehydrogenase has been solubilized from liver mitochondria and many of its properites described [10], early studies of choline dehydrogenase were hampered by the lack of a suitable assay, which may explain the apparent absence of the enzyme from guinea pig livers. With early assays in tissues with low choline dehydrogenase activity, the samples had to be incubated for long time intervals (> 1 hr) to obtain sufficient formation of the product. This resulted in a lack of steady-rate conditions as the substrate was depleted and as the inhibitory product (betaine aldehyde) accumulated. Furthermore, the studies of Rendina and Singer [10] on the effects of various electron acceptors on enzyme activity raise a question as to whether or not maximal enzyme activity was measured in early work because of the absence of an appropriate electron acceptor. All these problems may have led to erroneous conclusions about kinetics of the enzyme and

* Reprints: Dr. Dean R. Haubrich, Merck Institute for Therapeutic Research, West Point, PA 19486, U.S.A.
Present address: Sterling Winthrop Research Institute, Rensselaer, NY 12144, U.S.A.

about its biological properties. Furthermore, because the product of the oxidation of choline, betaine aldehyde, is further oxidized by betaine aldehyde dehydrogenase (EC 1.2.1.8) to betaine, many of the early studies in tissue homogenates and slices measured the activity of both enzymes.

Because of the renewed interest in choline as a pharmacologic agent, we have undertaken studies to elucidate the role of choline dehyrogenase in the regulation of choline metabolism. A sensitive radioisotopic assay that is specific for choline dehydrogenase was developed that permits initial velocities to be measured even when activity is low. The species and tissue distribution of the enzyme, as well as some of the properties of both the soluble and particulate enzyme, are reported.

METHODS

Radioisotopic assay for choline dehydrogenase. [^{3}H-Methyl]choline (obtained from the New England Nuclear Corp., Boston, MA, U.S.A.) was purified as follows: the solvent was evaporated from the sample under vacuum at 50–60°, and then the isotope was redissolved in a mixture of 0.17 N NaOH and 10% H_2O_2 and incubated for 1 hr at 24° to oxidize the trace amount of [^{3}H]-betaine aldehyde present in the sample. The entire sample was then subjected to paper electrophoresis at 1.5 kV for 1.5 hr in a pyridine– (0.3 M) formic acid (0.55 M) buffer at pH 4.5 (Miles High-Volt apparatus, Sussex, England) to isolate [^{3}H-methyl]choline from [^{3}H]-betaine. Radioactive choline was eluted from the paper, diluted with unlabeled choline to obtain the desired specific activity, and used within the same day. The incubation mixture for routine assays consists of the tissue homogenate (about 5 mg wet weight/ml) or solubilized enzyme, [^{3}H-methyl]choline (5 mM), phenazine methosulfate (PMS; 1 mM for soluble enzyme; 2 mM for mitochondria), and sodium phosphate (50 mM) of Tris buffer (50 mM; pH 8.0) in a final volume of either 15 or 25 μl. The reactions were performed for 15 min (except where indicated) at 37° in the dark to prevent nonenzymatic oxidation of PMS. At the end of the incubation period, NaOH (5 μl of 1 N) and H_2O_2 (10 μl of 30%) were added, and the samples were mixed and then incubated for 1 hr at room temperature to quantitatively convert [^{3}H]-betaine aldehyde to [^{3}H]-betaine. To isolate the product, the samples were subjected to ion exchange chromotography essentially as described previously [11]. The reaction mixture was transferred to a Pasteur pipette containing 1.0 ml of a 1:1 (v/v) slurry of BioRad AG 50W-X8 200–400 mesh resin for separation of [^{3}H]-choline from [^{3}H]-betaine. The resin had previously been converted from the hydrogen to the lithium form by stirring with 1 M LiOH, which was found to be essential to the quantitative elution of [^{3}H]-betaine. [^{3}H]-Betaine was eluted from the resin with 3 ml of water and collected into a counting vial for measurement of radioactivity by liquid scintillation spectrometry. All of the unreacted choline was retained on the ion exchange resin. The identity of the product and the extent to which [^{3}H]-betaine aldehyde was oxidized by H_2O_2 were confirmed by isolation of the product by paper electrophoresis as described above. Markers of choline, betaine and betaine aldehyde were identified by visualization with iodine vapors or Dragendorf's reagent. The migration rates during electrophoresis were 24, 20 and 2 cm/1.5 hr for choline, betaine aldehyde and betaine respectively. Blank values were less than 0.5 per cent of added radioactivity. Sufficient enzyme was added to convert 2–10 per cent of radioactive choline to betaine aldehyde. Results for all *in vitro* assays are averages of at least three determinations. Variability was always less than ±10 per cent of the mean.

Partial purification of choline dehydrogenase. Liver of rats was used as the starting material for purification of choline dehydrogenase, which was performed as described previously by Kimura and Singer [12]. Liver was homogenized in 0.25 M sucrose which contained K_2HPO_4 (0.01 M; pH 7.8), and then was centrifuged for 10 min at 600 *g* (r_{av} = 14.6 cm) using a Sorvall RC5 centrifuge with a No. 65A rotor. The supernatant fluid was centrifuged for 30 min at 3900 *g* (as above) to obtain the mitochondrial pellet, which was then washed twice with one-fourth the original volume of 0.25 M sucrose. The washed mitochondrial pellet was converted to an acetone powder by resuspending the tissue in a small volume of 0.25 M sucrose and immediately blending the suspension into 30–50 volumes of acetone at −10°. The precipitate was centrifuged, and the pellet was washed once with acetone (−10°), filtered, washed once with ether (−10°) and dried under vacuum. The acetone powder was stored at −20° in a desiccator.

To solubilize choline dehydrogenase, acetone powders were homogenized in 0.06 M glycine buffer (pH 10.3) and centrifuged for 20 min at 30,000 *g* (r_{av} = 7.7 cm) using a Beckman L-5-75 ultracentrifuge with Type 30 rotor. The pellet was resuspended in the same volume of sodium phosphate buffer (0.3 M, pH 7.6) and centrifuged again at 30,000 *g* for 20 min. The pellet was resuspended in half the original volume of 6 mM Tris phosphate buffer (pH 8.0) and incubated with 1 mg of *Naja naja* venom/100 mg protein (obtained from the Sigma Chemical Co., St. Louis, MO) at 30° for 40 min. The solubilized enzyme mixture was cooled and centrifuged at 105,000 g for 15 min. The supernatant fluid (solubilized choline dehydrogenase) was frozen in aliquots and stored at −20° where it was stable for at least 1 month. Sodium dodecylsulfate polyacrylamide gel electrophoresis revealed only four major bands between the molecular weights of 51,000 and 66,000. It has not been shown whether the enzyme is a single polypeptide chain or has subunits.

Other assays. The spectrophotometric assay for choline dehydrogenase was described previously by Singer [13]. Protein was measured as described by Lowry *et al.* [14], with bovine serum albumin (Sigma Chemical Co.), as standard. Unless indicated, all values are expressed in terms of protein concentration. Radioactive choline and betaine were isolated from tissues by a combination of high voltage paper electrophoresis and paper chromotography as described previously [7]. Endogenous choline was measured after electrophoretic isolation by an enzymatic assay using choline kinase [15]. The com-

pounds used *in vivo* did not interfere significantly in the assay for choline.

Animals. Most of the studies were performed using male Sprague–Dawley rats or female CF1 mice. Other animals used were guinea pigs (female; Charles River, Wilmington, MA), marmosets (*Saquinous Labiutus*; Primate Imports, Port Washington, NY), sheep (Merino cross breed, Quaker Farms, Quakertown, PA), dogs (mixed sex; Haycock Farms, Quakertown, PA), cats (mixed breed and sex; Quaker Farms), rabbits (New Zealand White; Buckshire Corp., Perkasie, PA), toads (female; *Buto Marinus*; Charles Chase, Miami, FL) and baboons (mixed sex, *Papiohamadryas*; International Exchange Agency, Ferndale, MI). Human tissue was obtained from accident victims and had been stored at −20° for more than 3 years.

RESULTS

Properties of partially purified choline dehydrogenase. Some of the properties of choline dehydrogenase were determined using the radioisotopic assay. As shown in Fig. 1, the rate of formation of betaine aldehyde was linear with respect to reaction time for at least 30 min when the incubation was performed in the light; however, the velocity declined sharply after 60 min of incubation in the light. In contrast, when the reaction was performed in the dark, linearity was achieved for at least 2 hr incubation time (Fig. 2) falling off slightly between 2 and 6 hr. This finding suggested that loss of activity with time occurred because of the loss of PMS (which is unstable in light) or the formation of an inhibitory product from PMS. In support of the latter hypothesis, we found that preincubation of the enzyme with PMS for 1 hr in the light prior to measurement of enzyme activity resulted in substantial loss of activity when the concentration of PMS was 2 mM or greater but not at a concentration of 1 mM or less (data not shown).

The velocity of the reaction was a linear function of the protein concentration up to 10 mg of soluble protein or 60 mg of mitochondria protein (Fig. 3). The rate of the reaction increased quickly as the pH of the medium was increased from 6 to 7, and tended to remain constant up to pH 9 (Table 1). Enzyme activity increased with temperature up to 37°, then

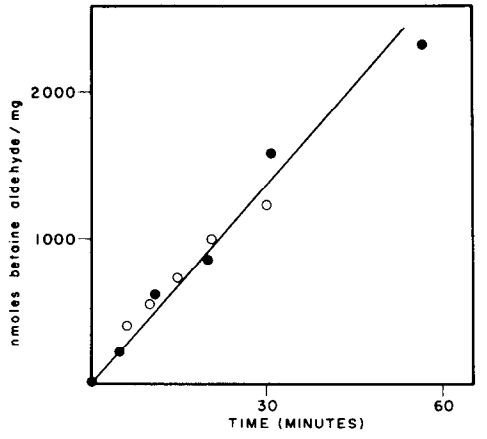

Fig. 1. Formation of betaine aldehyde plotted as a function of the reaction time using soluble (○) or mitochondrial (●) enzyme from rat liver. The reaction was performed in the light at 37°.

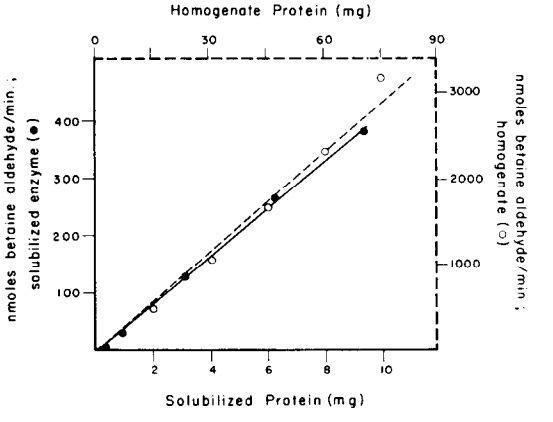

Fig. 3. Formation of betaine aldehyde plotted as a function of enzyme concentration using homogenates (○) or solubilized enzyme (●) from rat liver. Protein concentrations are mg/25 μl.

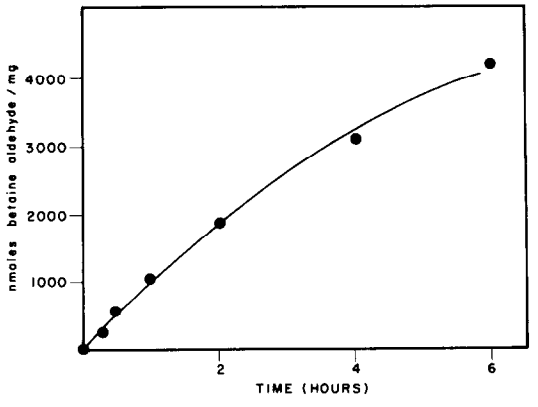

Fig. 2. Formation of betaine aldehyde plotted as a function of time when the reaction was performed in the dark at 24° using enzyme solubilized from rat liver.

Table 1. Effect of pH on velocity of choline dehydrogenase*

pH	Buffer	Velocity [nmoles/min·(mg protein)$^{-1}$]
6.0	Phosphate	4.95
6.5	Phosphate	24.1
7.0	Phosphate	38.0
7.2	Tris	35.2
7.5	Phosphate	40.6
8.0	Phosphate	38.2
8.0	Tris	35.6
8.5	Tris	35.2
9.0	Tris	38.5

* Rat liver was homogenized in various buffers. The concentration of each buffer during the assay was 50 mM. The samples contained 5 mg (wet weight) of tissue per ml, and were incubated for 15 min.

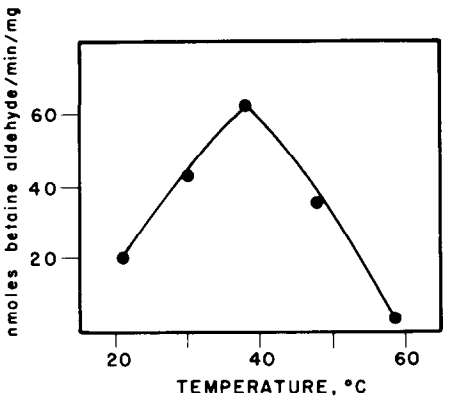

Fig. 4. Formation of betaine aldehyde plotted as a function of temperature using solubilized rat liver enzyme.

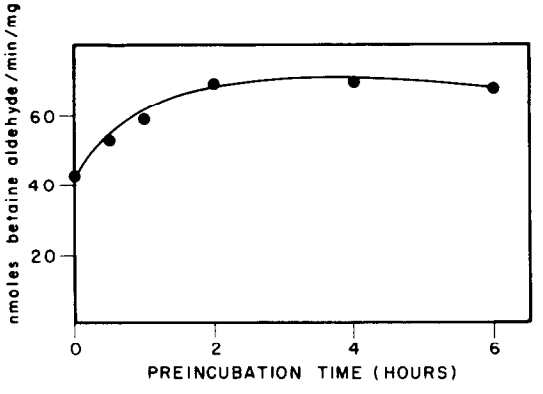

Fig. 5. Formation of betaine aldehyde by soluble rat liver enzyme plotted as a function of the preincubation time at 24°.

declined rapidly such that at 58° there was virtually no activity (Fig. 4). Furthermore, the soluble enzyme could be activated by incubation at room temperature for several hours (Fig. 5). These findings raise the possibility that the enzyme consists of subunits which are dispersed by either heating or incubation, thereby exposing additional active sites.

Dialysis of the soluble enzyme was performed in an effort to determine its requirement for a dissociable cofactor, but this treatment did not appreciably alter the activity (data not shown). Furthermore, addition to the enzyme of various naturally occurring electron acceptors had no effect on enzyme activity (Table 2). Only PMS and dichlorophenolindophenol (DCIP) stimulated the enzyme, with PMS having the greater potency. The solubilized enzyme was not appreciably affected by addition of 1 or 10 mM concentrations of ferrocyanide, ferricyanide, KCl, NaCl or $CaCl_2$. These results indicate that choline dehydrogenase does not require a loosely bound cofactor for activity, but do not exclude the possibility that choline dehydrogenase has a tightly bound cofactor such as FAD which resists removal by dialysis.

Kinetic constants were determined using the radioisotopic assay and were compared with those obtained with the spectrophotometric assay using the same enzyme preparation. As shown in Table 3, the radioisotopic method yielded a higher K_m and V_{max} for choline as compared to the spectrophotometric procedure. For PMS, the K_m was lower for the spectrophotometric assay as compared to the radioisotopic method, whereas the V_{max} values were essentially the same with the two methods. K_m values for choline and PMS were similar for both the soluble and the mitochondrial enzymes, whereas the V_{max} values were somewhat lower for both PMS and choline for the mitochondrial preparation as compared

Table 2. Effects of electron acceptors on choline dehydrogenase activity*

Electron acceptor	Concn (mM)	Enzyme preparation	Choline dehydrogenase (nmoles/min·mg^{-1})
Experiment I			
None		Solubilized	0
NAD	1.0	Solubilized	0
NADP	1.0	Solubilized	0
FAD	1.0	Solubilized	0
Coenzyme Q_0	1.0	Solubilized	0
Coenzyme Q_{10}	1.0	Solubilized	0.8
Experiment II			
PMS	0.1	Solubilized	26
PMS	0.5	Solubilized	63
PMS	1.0	Solubilized	67
PMS	2.0	Solubilized	68
PMS	5.0	Solubilized	57
PMS	10.0	Solubilized	45
Experiment III			
None		Mitochondria	0
DCIP	0.1	Mitochondria	10
DCIP	1.0	Mitochondria	11
DCIP	10.0	Mitochondria	6
PMS	0.1	Mitochondria	19
PMS	1.0	Mitochondria	39
PMS	10.0	Mitochondria	30

* Samples were incubated for 15 min with about 1 mg protein/ml.

Table 3. Kinetic constants for choline dehydrogenase*

	K_m (mM)			V_{max} [nmoles/min·(mg protein)$^{-1}$]		
	Radioisotopic		Spectrophotometric	Radioisotopic		Spectrophotometric
Substrate	Soluble	Mitochondria	Soluble	Soluble	Mitochondria	Soluble
Choline	5.7	5.3	1.4	77	34	49
PMS	0.1	0.1	0.8	63	42	59

* Choline dehydrogenase activity was determined under optimal assay conditions as described in Methods.

to the soluble enzyme. Lineweaver–Burk plots for choline were always linear over the entire range of concentrations. When the concentration of PMS was varied, however, nonlinear double-reciprocal plots were always obtained. The electron acceptor inhibited the soluble enzyme at a concentration above 2 mM and the mitochondrial enzyme above 1 mM (see Table 2).

Various analogs of choline were tested as potential inhibitors of choline dehydrogenase as shown in Table 4. The most potent compound was the product of the reaction, betaine aldehyde, with a K_i for the soluble enzyme of 0.6 mM. Other naturally occurring analogs were considerably less potent as inhibitors (K_i values > 10 mM). Two analogs of choline that do not occur naturally, dimethylaminoethanol and 2-amino-2-methylpropanol, were relatively potent inhibitors of choline dehydrogenase.

Properties in vivo *of choline dehydrogenase.* Various types of tissue homogenates were evaluated to determine conditions for measurement of optimal enzyme activity. In preliminary experiments, enzyme activity was found to be confined largely to the particulate fraction of rat livers. The highest activity in homogenates was achieved in Tris (50 mM) that contained sucrose (250 mM), with similar activity occurring whether or not the tissue was homogenized by hand using an all-glass homogenizer or with a Polytron homogenizer. Addition of PMS to rat liver homogenates stimulated enzyme activity about 50 per cent and was therefore used in all subsequent experiments.

Because of earlier reports that choline dehydrogenase was absent from some species, we have reassessed the distribution of enzyme activity in various tissues and species. As shown in Table 5, the enzyme was present in kidney and liver of all species tested. Rats, mice and toads had high activity in both tissues. In some species such as guinea pigs and humans, the greatest activity was observed in kidneys. In other tissues, the enzyme either could not be detected (muscle and fat) or was barely detectable (blood, spleen and heart). Because the concentration of free choline in tissues was greater than 0.5 nmole/mg protein (Table 6), choline from the tissues would not have diluted the [^{3}H]-choline by more than 1 per cent even if a 2-fold postmortem increase in choline had occurred during the assay. The failure of earlier investigators to detect enzyme activity was apparently not the result of differences in the age of animals. Choline dehydrogenase was present 1 day after birth, as shown in Fig. 6, and reached its peak activity in the liver at 1 week of age and in the kidney at 4 weeks.

Table 4. Inhibition of choline dehydrogenase by analogs of choline*

Compound		K_i (mM) Soluble	K_i (mM) Mitochondria
$(CH_3)_3\overset{\oplus}{N}CH_2CHO$	Betaine aldehyde	0.6	1.4
$(CH_3)_2NCH_2CHOH$	Dimethylaminoethanol	1.8	3
$H_2N-C(CH_3)_2-CHOH$	2-Amino-2-methylpropanol	1.4	
$(CH_3)_3N$	Trimethylamine		10
CH_3NCH_2CHOH	Methylaminoethanol		20
$(CH_3)_3\overset{\oplus}{N}CH_2CH_2OPO_3H_2$	Phosphorylcholine		23
$(CH_3)_3\overset{\oplus}{N}CH_2CH_2$—[CDP]	CDP–choline		50
$(CH_3)\overset{\oplus}{N}CH_2CH_3OCOCH_3$	Acetylcholine		50
$(CH_3)_3\overset{\oplus}{N}CH_2COOH$	Betaine		>50

* All compounds were competitive inhibitors of choline dehydrogenase, as determined from a Dixon plot using three concentrations of choline, three to five concentrations of inhibitor, and duplicate determinations for each point.

Table 5. Choline dehyrogenase in various species and tissues*

Species	N	Tissue	Choline dehydrogenase (nmoles/min·mg^{-1} ± S.D.)
Toad	3	Liver	33.1 ± 3.9
		Kidney	10.4 ± 1.0
Rat	3	Liver	22.2 ± 0.4
		Kidney	17.1 ± 1.9
		Brain	0.27 ± 0.37
		Lung	0.15 ± 0.06
		Blood	0.004 ± 0.007
		Spleen	0.001 ± 0.002
		Heart	0.001 ± 0.002
		Muscle	Not detectable
		Fat	Not detectable
Mouse	8	Liver	9.1 ± 2.1
		Kidney	8.3 ± 1.2
Cat	3	Liver	7.3 ± 2.3
		Kidney	4.2 ± 2.4
Dog	4	Liver	4.2 ± 2.5
		Kidney	3.4 ± 0.4
Monkey	3	Liver	3.0 ± 2.0
		Kidney	2.1 ± 1.6
Sheep	4	Liver	1.5 ± 0.3
		Kidney	0.45 ± 0.12
Human	2	Liver	1.3 ± 0.2
		Kidney	7.1 ± 0.1
Rabbit	2	Liver	1.0 ± 0.2
		Kidney	2.4 ± 0.5
Baboon	3	Liver	0.28 ± 0.11
		Kidney	1.17 ± 0.22
		Spleen	0.067 ± 0.076
		Lung	0.045 ± 0.051
Guinea pig	3	Liver	0.15 ± 0.13
		Kidney	1.79 ± 0.39
		Brain	Not detectable
		Lung	Not detectable
		Blood	0.058 ± 0.051
		Spleen	0.017 ± 0.029
		Heart	0.021 ± 0.020
		Skeletal muscle	Not detectable
		Fat	Not detectable
		Duodenum	0.38 ± 0.08

* Enzyme activity was measured in homogenates (5 mg wet weight of tissue/ml) using boiled tissue as blank.

In order to compare the kinetic parameters of choline dehydrogenase *in vitro* with the *in vivo* conditions under which choline is oxidized, the endogenous level of free choline and the rate of oxidation of radiolabeled choline were measured in rat liver. As shown in Table 6, the endogenous concentration of free choline was 50 nmole/g of wet tissue (tissue was 10% protein) or greater than 50 μM. The rate of oxidation of [^{3}H-methyl]choline was 0.3 nmole/min·(mg protein)$^{-1}$ calculated using the isotope dilution equation $\frac{[^3H]\text{ betaine}}{[^3H]\text{ choline}} \times$ endogenous choline = nmoles/min·mg^{-1}. [^{3}H]Betaine aldehyde was virtually undetectable in the tissue, possibly because it is oxidized to betaine by betaine aldehyde dehydrogenase as quickly as it is formed.

To determine if choline dehydrogenase is involved in regulation of the concentration of choline in tissues, two inhibitors of the enzyme were administered to mice and the concentration of free choline was measured. As shown in Table 7, treatment with either dimethylaminoethanol or 2-amino-2-methylpropanol caused a marked increase in the concentration of choline in both liver and kidney.

Table 6. Rate of *in vivo* oxidation of choline in rat liver*

Tissue	Endogenous choline (nmole/mg ± S.D.)	Radioactive choline (dpm/mg ± S.D.)	Radioactive betaine (dpm/mg ± S.D.)	Rate of oxidation (nmole/min·mg^{-1} ± S.D.)
Liver	0.5 ± 0.2	4660 ± 180	2740 ± 550	0.29 ± 0.04

* Rats were decapitated 1 min after intravenous administration of [methyl-^{3}H]choline (1.4 mCi/kg; 0.6 μmole/kg). Tissues were removed within 45 sec and frozen immediately in acetone with dry ice. Values are per mg protein; N = 6.

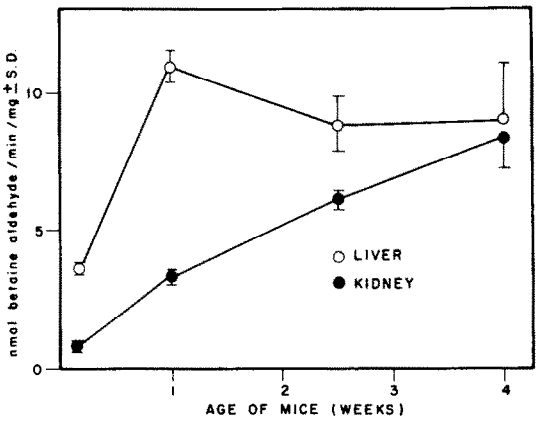

Fig. 6. Formation of betaine aldehyde plotted as a function of age in mouse kidneys and livers.

Table 7. Effect of choline dehydrogenase inhibitors on the *in vivo* concentration of choline in mice*

Inhibitor	Choline (nmoles/g† ± S.D.) Kidney	Liver
Control	118 ± 28	55 ± 18
Dimethylaminoethanol	465 ± 103‡	97 ± 14‡
2-Amino-2-methylpropanol	411 ± 26‡	145 ± 40‡

* Mice were decapitated 2 hr after treatment with 5 nmoles/kg (i.p.) of each compound and tissues were removed and immediately frozen in acetone on dry ice.

† Wet weight of tissue.

‡ Different from control, $P < 0.05$ (Dunnett's *t*-test).

DISCUSSION

The assay used in the present study of choline dehydrogenase is more specific than some of the earlier methods in which oxygen production or dye reduction was measured because there is no interference in the present assay by betaine aldehyde dehydrogenase, which is present in whole homogenates of tissue and, to some extent, in the particulate fraction. In addition, choline analogs tested as potential inhibitors of choline dehydrogenase, even though some of them are substrates for the enzyme [16], do not interfere, and therefore accurate inhibitory constants can be determined. The sensitivity of the present assay is also greater than previous methods, and therefore the incubations can be performed for a shorter time intervals even when enzyme activity is low (as in some tissues), thereby eliminating the possibility of depletion of the substrate or accumulation of the inhibitory product. Furthermore, when the substrate concentration is reduced to perform kinetic studies, the sensitivity does not change, or can actually increase if the amount of radioactive choline in the sample is kept constant, because more radioactive betaine aldehyde is formed without a change in the blank value.

Using this assay, choline dehydrogenase was found to occur in all species examined and was present in highest concentrations in either the kidney or liver. The higher levels in kidneys of some species, together with the use of relatively insensitive assays, may account for the inability of early investigators to detect the enzyme in liver of some species such as guinea pigs [8] even though large amounts of radioactive betaine were found when labeled choline was administered to this species [7].

The properties of the enzyme determined with the present assay are in good agreement with the results of Rendina and Singer [10] using a manometric assay with phenazine methosulfate to measure activity in a solubilized preparation. The K_m of about 5 mM for choline is consistent with a value of 7 mM reported by Rendina and Singer [10] but higher than values reported by others (~ 1 mM) [17]. Choline dehydrogenase was not stimulated by addition of NAD or NADP, in contrast to other results [18], presumably because betaine aldehyde dehydrogenase, which does not require these cofactors, did not interfere in the present assay. The enzyme requires phenazine methosulfate for maximal activity and has a pH optimum in the range between 7.0 and 9.0. Other investigators who have measured activity at a lower pH [9, 18] and without the addition of an appropriate electron acceptor have presumably not measured all of the enzyme activity. Thus, in most early work on the biological properties of the enzyme, conditions for the assay were not always optimal.

The results of our kinetic studies on the solubilized and the particulate enzyme were compared with the concentration of endogenous choline and its rate of oxidation *in vivo*. The rate of oxidation *in vivo* [0.29 nmole/min · (mg protein)$^{-1}$] was markedly less than the V_{max} for the enzyme [34 nmoles/min · (mg protein)$^{-1}$ in mitochondria, 22 nmoles/min · (mg protein)$^{-1}$ in rat liver homogenates]. This difference was not likely the result of unphysiologic stimulation of the enzyme by the addition of PMS to the *in vitro* assay system, because in homogenates the activity was only stimulated by 50 per cent. It may be explained, however, by the relatively low concentration in liver of free choline *in vivo*, which is about 60 μM (calculated from a value of 50 nmoles/g of wet tissue and assuming that liver is 80% water) as compared to its much higher K_m (> 5 mM). Thus, assuming uniform distribution of choline, the enzyme would not be saturated by its substrate under physiologic conditions. This suggests that the rate of oxidation of choline *in vivo* will depend upon the concentration of free choline such that a large increase in the tissue concentration would be attenuated by "spill over" into the oxidative pathway. Thus, choline dehydrogenase may be partially responsible for the rapid turnover of free choline in the body [7], and for the inability to induce a sustained elevation of free choline in humans even when large doses of choline are administered repeatedly [19]. The hypothesis that choline dehydrogenase may be involved in the control of free choline levels is further supported by the present finding that two inhibitors of choline dehydrogenase, when administered to mice, caused an increase in the concentration of free choline in liver and kidney. In other studies, deanol has been shown to induce an increase in the concentration of choline in brain [2] and blood [20], and

administration of 2-amino-2-methylpropanol caused an increase in the level of free choline in brain. Thus, choline dehydrogenase in the periphery may regulate the availability of choline in the blood and, ultimately, in the brain where choline may serve to modulate the activity of central cholinergic neurons.

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