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A POSSIBLE MECHANISM FOR THE INCREASED OXIDATION OF CHOLINE AFTER CHRONIC ETHANOL INGESTION *

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Summary

An attempt has been made to determine the location of the site at which the metabolism of ethanol interacts with that of choline to produce an increase in the oxidation of choline. The first enzyme in the oxidation pathway for choline, choline dehydrogenase, was assayed using a newly developed spectrophotometric assay and freshly isolated intact rat liver mitochondria. No changes were observed in either the 'apparent' V or the 'apparent' K_m values of choline dehydrogenase for choline after ethanol ingestion. However, when the choline oxidase system was assayed, a 28% decrease in 'apparent' K_m for choline and a 53% increase in 'apparent' V was observed. The effects of ATP on choline oxidase were studied further, and a 29.4% decrease was observed in mitochondrial ATP levels from freshly isolated mitochondria from the ethanol-treated rats. In vitro aging of mitochondria further decreased the level of ATP, and the rate of decrease was considerably faster during the first hour in the mitochondria from the ethanol-treated animals. The decreases in ATP from both control and experimental mitochondria were accompanied by increases in choline oxidase activity. The initial decrease in ATP was correlated with an increase in mitochondrial ATPase activity which may be related to an increase in mitochondrial Mg^{2+}. Because chronic ethanol ingestion has resulted in decreased oxidation rates of succinate and β-hydroxybutyrate while at the same time increasing the oxidation rates of choline, the studies reported here suggest that the effect of chronic ethanol ingestion is primarily on a step that is unique to choline and which probably exists prior to the electron transport chain.

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Introduction

Research has shown that the actual metabolism of ingested ethanol increases the uptake of choline by the liver [1]. Hydrogen acceptors have been used to relate the increased choline uptake to the NADH generated during ethanol metabolism [2]. Furthermore, choline oxidase inhibitors have been shown to depress the hepatic uptake of choline [1]. These data suggest that choline uptake is related to the oxidative pathway of choline metabolism. A previous report from this laboratory has supported this contention by showing that both the acute and chronic ingestion of ethanol increased the rate of choline oxidation in liver mitochondria [3].

The oxidation of choline by mitochondria involves a rather complicated system of enzymes and electron transfers. Choline dehydrogenase, the first enzyme in the oxidative pathway of choline catabolism, converts choline to betaine aldehyde and reduces a flavoprotein via FAD conversion to $FADH_2$. Singer [4] has suggested that the reduction of the flavoprotein can be coupled to dichlorophenol-indophenol with phenazine methosulfate. Thus, a spectrophotometric assay could be developed to monitor the reduction of dichlorophenol-indophenol. The reduced phenazine methosulfate also could be coupled directly with molecular oxygen, and the dehydrogenase could be assayed polarographically. In the complete choline oxidase reaction, the electrons flow from choline through the flavoprotein into the electron transport chain and on to molecular oxygen. Thus, the oxidase can be easily monitored polarographically [3]. To determine the location of the ethanol effect on choline oxidation, it was necessary to measured the activity of the primary dehydrogenase enzyme, choline dehydrogenase, apart from that of total choline oxidase system. Thus, by comparing the results from the direct analysis of choline dehydrogenase to those from the analysis of choline oxidase, an attempt was made to determine where the metabolism of ethanol interacts with that of choline to produce the increase observed in choline oxidation after chronic ethanol ingestion.

It has been suggested [5,6] and further supported [7] that adenine nucleotides exert control over the oxidation of choline. All three adenine nucleotides (ATP, ADP and AMP) are inhibitory to choline oxidation [5,6]. However, de Ridder and van Dam [7] have shown that when a mixture of ATP with either one of the other two nucleotides or with both was incubated with isolated choline dehydrogenase, the influence of ATP was dominant. It should be pointed out that the assay technique used in obtaining the above data [7] was essentially that described for choline oxidase above. Therefore, the effects of the nucleotides may be related to the electron transport chain as well as to choline dehydrogenase. Further, Wilken et al. [6] suggested that the site of the ATP or ADP effect was somewhere before cytochrome *c* reduction.

Because chronic ethanol ingestion results in decreased levels of hepatic adenine nucleotides [8,9], choline oxidation has been studied in an attempt to distinguish between the effects of ATP directly on the dehydrogenase enzyme and the effects of ATP exerted via the electron transport chain.

Materials and Methods

Male Long-Evans rats weighing 140—160 g were obtained from Simonsen Labs, Gilroy, Calif. They were paired by weight and then pair-fed a low-fat liquid diet [3] for 77—99 days. The ethanol-treated animals weighed 396 ± 7.4 g at the time of killing, and the controls weighed 373 ± 15.3 g. This represents an average daily weight gain of 2.9 g/day for the ethanol-treated and 2.7 g/day for the control animals. The liver weights were 38.5 ± 0.7 g/kg body weight for the ethanol-treated and 32.7 ± 3.2 g/kg body weight for the control animals. All of these values represent the mean ±S.E.M. In the experimental diet, ethanol was added so that it would replace 36% of the carbohydrate calories. Richter tubes were used for daily measurement of diet consumption. During the last week prior to killing, the daily intake of ethanol was about 16 g/kg per day. Blood ethanol values were not obtained. Rats were killed by decapitation, and their livers were immediately removed and placed in ice-cold 0.25 M sucrose (pH 7.4). Mitochondria were isolated according to the procedure of Chappell and Hansford [10] with modifications as described elsewhere [3].

Sonication experiments involved the use of the 100% setting on a Biosonik IV sonicator. In the freeze-thaw experiments, the mitochondria were frozen slowly by placing them at −18°C. The frozen suspension was then allowed to slowly thaw to room temperature. Experiments using deoxycholate involved exposing mitochondrial suspension to 0.8% deoxycholate for varying lengths of time. Spectral measurements were made with a Beckman Model 25 recording spectrophotometer. Oxygen utilization was measured polarographically with a Clark oxygen electrode.

Measurements of the total choline oxidase system were performed polarographically. The total reaction mixture contained 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris-HCl (pH 7.4), 0.005 M potassium phosphate, and 0.05 M choline. When betaine aldehyde oxidation was measured, its concentration was 0.05 M. The reaction was initiated by adding 0.05 ml of a mitochondrial suspension which contained about 1.5—2.0 mg protein. Maximal rates of choline oxidase activity were obtained by including 1 mM $CaCl_2$ in the reaction mixture. A temperature of 33°C was determined to be optimal for the reactions. Protein concentrations were determined by the procedure of Lowry et al. [11].

To determine the kinetic parameters for choline dehydrogenase, a 1.0 ml reaction was used with either the polarographic or spectrophotometric assays. All reaction mixtures contained 0.23 M mannitol, 0.07 M sucrose, 0.02 M Tris-HCl (pH 7.4), 0.005 M potassium phosphate, 0.002 M KCN, 0.1 M semicarbazide, and $1 \cdot 10^{-5}$ M antimycin A. The spectral assay included 1.0 mg phenazine methosulfate and either $3.06 \cdot 10^{-5}$ M 2,6-dichlorophenol-indophenol or 1.25 mg cytochrome *c*. The polarographic assay included only 1.0 mg phenazine methosulfate. The kinetic studies were done using 2,6-dichlorophenol-indophenol ($3.06 \cdot 10^{-5}$ M) with varying concentrations of phenazine methosulfate (0.5, 1.0, 1.5, 2.0, and $2.5 \cdot 10^{-4}$ g, respectively), and choline (0.3, 0.6, 0.9, 1.2, and 1.5 mM). When $CaCl_2$ was added, 1.0 mM was used. These values represent final concentrations in the reaction mixture. The reduction of cyto-

chrome *c* and dichlorophenol-indophenol was followed at 550 and 600 nm, respectively, and extinction coefficients of $1 \cdot 18.5$ and $21 \cdot 10^3$ l/mol per cm were used, respectively, to convert back to choline equivalents. Three different concentrations of protein were used for each mitochondrial preparation: 0.1—0.3 mg for the spectral assay and 0.8—2.4 mg for the polarographic assay.

The reaction mixture for [^{14}C]choline (9.98 μCi/mmol) uptake studies was the same as that employed in the choline oxidase study. The actual uptake of [^{14}C]choline was measured after rapid filtration of aliquots of the mitochondrial suspension through two Millipore filters (pore size 0.45 μM), essentially as described by Rasmussen et al. [12]. Aliquots of the suspension were pipetted into a 5-ml Luer-Lok syringe, containing a Swinny adapter holding the Millipore filters. The suspension was filtered by applying moderate pressure to the plunger of the syringe. The entire sampling operation was complete within 5 s. Aliquots of filtrates were counted for ^{14}C in a Beckman liquid scintillation system. The liquid scintillation cocktail was a 2 : 1 mixture of toluene and Triton X-100 which contained PPO (5.5 g/l) and POPOP (0.1 g/l).

Concentrated mitochondrial suspensions (40—50 mg/ml) were kept at 0°C in an ice bucket for one of the aging studies. Aliquots of the aging mitochondrial suspension were removed at 10 min and centrifuged for 10 min at 12 000 rev./min (Beckman JA-20 rotor). To 1.0 ml of the supernatant, 1.0 ml 5% trichloroacetic acid, 0.5 ml 0.1 M acetate buffer (pH 5.2) and 60 mg norite (acid-washed) were added. This mixture was allowed to sit in an ice bucket for 1 h with occasional shaking and then centrifuged. To elute the adenine nucleotides, the norite was extracted 3 times with an aqueous solution of 10% pyridine and 50% ethanol. The absorbance at 260 nm was determined on this extract. Norite was determined to have a 94% binding efficiency of adenine nucleotides under these experimental conditions.

To determine ATP concentrations, aliquots of the aging mitochondrial suspension were removed at various time points and injected into three volumes of boiling water. The samples were allowed to heat at 100°C for 10 min and then centrifuged for 10 min at 12 000 rev./min (Beckman JA-20 rotor). ATP concentrations were determined on the supernatants using luciferase [13] and a Packard scintillation counter.

Mitochondrial ATPase activity was determined in a reaction mixture which contained in a final concentration: 80 mM NaCl, 10 mM succinate, and 5 mM Tris-HCl (pH 7.4), 8.4 mM ATP, 1.5 mg/ml mitochondrial protein and, when included, 1 mM $CaCl_2$, 10 mM $MgCl_2$ or 75 μM dinitrophenol. These conditions are similar to those used by Gear [14]. A 1.5 ml reaction was stopped with 0.25 ml 2.5 M perchloric acid and neutralized with 2.5 M KOH. After centrifugation, the supernatant was analyzed for inorganic phosphate according to the procedure of Eibl and Lands [15].

Mitochondrial levels of magnesium were determined with an atomic absorption spectrophotometer (Beckman 1300) equipped with an Autolam Burner (100410) and coupled to a Beckman DU spectrophotometer. Aliquots of mitochondrial suspensions were placed in a muffle furnace at 100°C overnight followed by 4 h at 450°C. The dry ash was extracted with 1% HCl containing 1% La_2O_3. The amount of magnesium present in the mitochondria was then determined using a multi-element lamp.

Bovine serum albumin (Fraction V) was defatted by isooctane extraction according to the procedure of Goodman [16].

Phenazine methosulfate, 2,6-dichlorophenol-indophenol, choline chloride, semicarbazide, cytochrome *c*, mannitol and antimycin A were purchased from Sigma Chemical Company. Ultrapure sucrose and Tris were purchased from Schwarz-Mann. All other compounds and reagents were reagent grade materials.

Results

When freshly isolated mitochondria were used, maximum polarographic rates for choline dehydrogenase required either physical or chemical treatment of the mitochondria. Fig. 1 shows that maximum activity was reached after 50 s of sonication, 2—3 times freezing and thawing or 60 s exposure to deoxycholate. However, the inclusion of 1 mM $CaCl_2$ proved to be the most convenient for increasing the reaction rate to the same maximal level as that found in the damaged mitochondria. As can be seen in Fig. 1, the inclusion of $CaCl_2$ allowed a maximum rate of activity to be attained even when only fresh mitochondria were used.

A more sensitive spectrophotometeric assay was developed, but because the reduced phenazine methosulfate was insoluble in aqueous media, the reoxidation of phenazine methosulfate was coupled with either cytochrome *c* or dichlorophenol-indophenol. Mechanical or chemical treatments also were required to obtain maximal reaction rates, and curves similar to those shown in

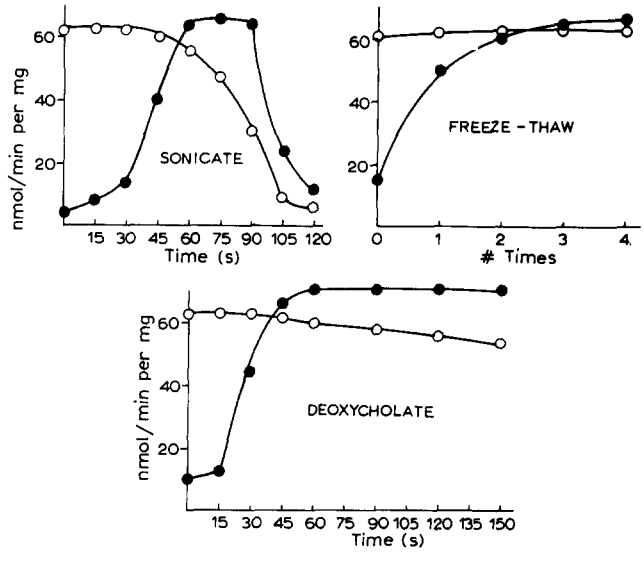

Fig. 1. Effect of Ca^{2+} on the polarographic measurement of choline dehydrogenase in intact mitochondria. Fresh liver mitochondria were prepared as described [3,10]. Oxygen consumption was measured polarographically, and the activity was converted to nmol choline oxidized/min per mg mitochondrial protein. The reaction mixture is described in Materials and Methods. Choline concentration was 0.05 M, and the phenazine methosulfate concentration was 1.0 mg. The reaction was initiated by adding 0.05 ml of a mitochondrial suspension containing 1.5—2.0 mg protein. The final volume was 1.0 ml, and the temperature was 33°C. The effect of 1.0 mM $CaCl_2$ (○) was compared to the absence of Ca^{2+} (●).

Fig. 1 were obtained. After the fourth freeze-thaw cycle, after a 2 min treatment with deoxycholate, after a 1 min sonication, or after the addition of 1 mM $CaCl_2$, the following rates of reaction were obtained: 76.0, 76.8, 72.4, and 76.2 nmol choline oxidized/min per mg mitochondrial protein, respectively.

Utilizing the spectral assay, 1 mM $CaCl_2$ and 0.1 mg phenazine methosulfate, the optimal concentration of cytochrome *c* and dichlorophenol-indophenol was 125 mg/ml and $3.06 \cdot 10^{-5}$ M, respectively. Both the spectral and polarographic assays were linear with protein concentration (spectral to 0.9 mg; polarographic to 2.5 mg).

Using [^{14}C]choline and determining the uptake rates by microfiltration techniques, mitochondria were shown to accumulate 50.02 ± 0.25 nmol choline/min per mg protein in the absence of $CaCl_2$ and 49.94 ± 1.3 nmol choline/min per mg protein in the presence of $CaCl_2$. The above numbers were determined during the first 5-s interval which represents the shortest time in which the filtration could be accomplished. Hence, it would appear that mitochondria take up choline at the same rate either in the presence or absence of $CaCl_2$.

A study of the kinetic parameters (V and K_m) was used to determine the effects of chronic ethanol feeding on choline dehydrogenase. Liver mitochondria were isolated and studied from each of 13 pairs of isocalorically fed rats. Only one pair of rats was killed on any given day. Three different concentrations of mitochondrial protein were used for each preparation, and each concentration of protein was studied at five different concentrations of the variable substrate, phenazine methosulfate, and five different concentrations of the fixed substrate, choline. The data obtained from this study were plotted via Lineweaver-Burk and resulted in a series of parallel linear lines for the five choline concentrations. These initial 1/v versus 1/s plots were then replotted using the 'y' intercepts and the reciprocal concentration of the fixed substrate, choline. These replots gave rise to the 'apparent' V for choline dehydrogenase and the 'apparent' K_m for choline. Table I presents the results of these determinations.

When kinetic data from the chronic ethanol-treated animals were compared to the controls, no difference was observed in choline dehydrogenase activity. This was true when either the polarographic or spectrophotometric assay was used. However, when the total choline oxidase system was measured, i.e., electrons flow from choline dehydrogenase through the electron transport system to oxygen, oxygen consumption in the mitochondria from the ethanol-treated animals was increased only slightly in the absence of added $CaCl_2$. The addition of 1 mM $CaCl_2$ increased the 'apparent' V in the control mitochondria 43.8%, whereas, in the mitochondria from ethanol-treated animals, there was a 97.6% increase in choline oxidase activity. Also, it was observed that 14 days after ethanol withdrawal the choline oxidase V value returned to control values.

Table I also compares the 'apparent' K_m for choline. Once again, there appeared to be no difference between the ethanol-treated and control animals. However, the 'apparent' K_m for choline in the oxidase system was decreased 22.7% by ethanol ingestion in the absence of $CaCl_2$ and 27.5% in the presence of $CaCl_2$. The addition of $CaCl_2$ to the choline oxidase system decreased the 'apparent' K_m for choline only slightly. 14 days after ethanol withdrawal, the

TABLE I

EFFECT OF CHRONIC ETHANOL INGESTION ON THE 'APPARENT' V FOR CHOLINE DEHYDROGENASE AND CHOLINE OXIDASE AND ON THE 'APPARENT' K_m FOR CHOLINE IN BOTH REACTIONS

The reaction mixtures for both choline dehydrogenase and choline oxidase are described in Materials and Methods. The 'apparent' K_m and 'apparent' V were determined by replots of 1/v versus 1/s data. Each number represents the mean ± S.E.M. from 13 pairs of animals. The reaction temperature was 33°C except in the polarographic measurement of choline dehydrogenase (phenazine methosulfate). The temperature was lowered to 24°C to ensure sufficient levels of oxygen to reoxidize the reduced phenazine methosulfate. Cytochrome C could substitute for dichlorophenol-indophenol, and results identical to those shown for the spectrophotometric assay were obtained.

Enzyme	Assay	Electron acceptor	'Apparent' V (nmol O_2/min per mg protein)		'Apparent' K_m (mM)	
			Control	Ethanol	Control	Ethanol
Dehydrogenase	Spectrophotometric	Phenazine methosulfate + dichlorophenol-indophenol	35.3 ± 0.1	35.0 ± 0.3	0.69 ± 0.02	0.66 ± 0.02
Dehydrogenase	Polarographic	Phenazine methosulfate	35.8 ± 0.4	35.2 ± 0.2	0.74 ± 0.02	0.74 ± 0.02
Oxidase	Polarographic	Electron transport system	11.4 ± 0.4	12.7 ± 0.4	1.19 ± 0.01	0.92 ± 0.01 *
Oxidase + Ca^{2+}	Polarographic	Electron transport system	16.4 ± 0.2	25.1 ± 0.3 *	1.09 ± 0.02	0.79 ± 0.02 *

* When compared with control values, these values were statistically different at the $P < 0.02$ level or better. A paired t test was used.

K_m for choline in the oxidase system returned to control values. It was concluded from these data that ethanol ingestion did not affect choline dehydrogenase and that the ethanol effect must be related to either the electron transport system or the coupling of the dehydrogenase to the electron transport system.

Because neither the spectrophotometric nor polarographic assays could distinguish between choline and betaine aldehyde dehydrogenase, the kinetic parameters also were determined in the presence of semicarbazide. It had no effect on the 'apparent' K_m for choline and caused less than a 5% decrease in the 'apparent' V for the choline-phenazine methosulfate reaction in either the control or experimental mitochondria. The oxidation of betaine aldehyde was measured polarographically in the control and ethanol-treated mitochondria in the absence of $CaCl_2$ (21.0 ± 0.85 and 21.24 ± 1.54 nmol O_2/min per mg, respectively) and in the presence of $CaCl_2$ (2.07 ± 0.10 and 2.07 ± 0.08 nmol O_2/min per mg, respectively). The inclusion of 0.1 M semicarbazide in the polarographic assay of betaine aldehyde oxidation (presence and absence of $CaCl_2$) totally prevented oxygen consumption rates.

It has been reported [7] that all three adenine nucleotides (ATP, ADP, AMP) have a direct effect on both the binding of choline and the rate of choline oxidation when studied in the intact oxidase system. When different levels of ATP (0.13—0.65 mM), ADP (0.13—0.64 mM) or both were added in the presence of oligomycin (1.7 μg/mg protein) to either the polarographic or spectral assay for choline dehydrogenase, no effect was observed on either V or K_m (data not shown). These results were observed for both the control and ethanol-treated animals.

In addition to studying the effects of direct nucleotide addition, the correlation between the nucleotides present in the isolated mitochondria and choline oxidase was determined. Aging the mitochondria proved to be an excellent means to alter ATP concentration. These studies showed that aging mitochondria at either 0 or 15°C resulted in the leakage of material absorbing at 260 nm. At 0°C there was a 60% increase in leakage of this material from the ethanol-treated mitochondria (0.016 A units/10 min) compared to the controls (0.010 A units/10 min). At 15°C there was a 61% increase in this leakage from the ethanol-treated mitochondria (0.132 A units/10 min) compared to the controls (0.082 A units/10 min). The amount of A_{260nm} leakage was the same even when defatted bovine serum albumin (Fraction V) was included in the aging medium. This may represent a gradual breakdown of ATP and leakage of ADP and/or ATP from the mitochondria.

When choline was added to a fresh mitochondrial suspension in the absence of Ca^{2+}, a slow rate of oxidation occurred for a finite period of time. This slow reaction rate then was followed by a fast reaction rate. In keeping with the nomenclature of Kagawa et al. [5], the slow rate represents the 'controlled' rate and the fast rate the 'uncontrolled' rate. Further, the length of time between the addition of choline and the onset of the 'uncontrolled' rate represents the lag time. In a separate experiment, the effects of mitochondrial aging on the choline oxidase system were studied. The mitochondria from the ethanol-treated animals oxidized choline only slightly faster than their controls when the 'controlled' phase of oxidation was measured (Table II). During

TABLE II

THE EFFECTS OF IN VITRO AGING ON CHOLINE OXIDATION AND ATP CONCENTRATION IN CONTROL AND ETHANOL-TREATED RAT LIVER MITOCHONDRIA

Choline oxidation rates and ATP concentrations of rat liver mitochondria were determined as described in Materials and Methods. $CaCl_2$ was not added to the reaction mixture for choline oxidase. The lag time represents the time required for the mitochondria to exhibit the 'uncontrolled' phase of oxidation. Each value represents the mean from 5—6 animals.

Aging (time/temperature)	'Controlled' rate (nmol O_2/min per mg)		Lag time (min)		'Uncontrolled' rate (nmol O_2/min per mg)		ATP (nmol/mg)	
	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
0 min/0°C	11.4	12.7	20	10	None reached	19.2	7.8	5.5
60 min/0°C	11.4	12.7	15	5	14.3	26.2	6.7	2.3
180 min/0°C	11.4	—**	11	—*	19.0	29.4	5.2	1.9
250 min/0°C	11.4	—**	9	—*	21.4	29.9	4.5	1.6

* No lag time was observed.

** No 'controlled' rate was observed; the 'uncontrolled' rate started immediately.

aging, a progressive decrease was observed in the lag time for the onset of the 'uncontrolled' phase of choline oxidation in mitochondria from the control animals. The mitochondria from the ethanol-treated animals exhibited a much shorter lag time than controls, and after 3 h of aging, no 'controlled' phase of oxidation was observed. When comparing the 'uncontrolled' phase of choline oxidation, the oxidation rates of the mitochondria from the ethanol-treated animals were increased 83, 55, and 40% after 60, 180, and 250 min of aging at 0°C, respectively. Because we had observed an increased leakage of A_{260nm} material, these data in Table II may be suggesting a decrease in the adenine nucleotide, specifically ATP, control over choline oxidase.

To determine if there was a relationship between choline oxidation and mitochondrial ATP, the ATP content of each mitochondrial preparation was determined at each of the time points from the previous study (Table II). Freshly isolated mitochondria from the ethanol-treated animals exhibited a 29% decrease in ATP content. At each time point during the aging process either 0 or 15°C), there was a 60—70% decrease in mitochondrial ATP content in the ethanol-treated animals when compared to controls.

It has been shown that ATP, ADP and AMP exert inhibitory effects on choline oxidation [5,7]. Mixtures of these nucleotides showed that ATP dominated the effects [7]. Therefore, if ATP has the controlling effect on choline oxidation, then there should be a relationship between mitochondrial ATP content and choline oxidase. A plot of all 'uncontrolled' rates (Table II) of choline oxidation versus mitochondrial ATP from both the control and ethanol-treated animals (Fig. 2) supports the contention that an inverse relationship exists between ATP and choline oxidation. Data from both control and ethanol-treated animals were used to construct Fig. 2. It is interesting to note that data from the ethanol-treated animals lie to the right. Extrapolation of this linear curve to zero ATP concentration gave a maximum rate of choline

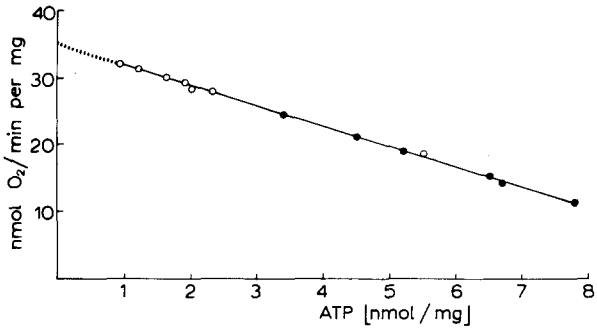

Fig. 2. Relationship of mitochondrial concentration and choline oxidation rates. Both the rates of choline oxidation and the concentration of mitochondrial ATP were determined as described in Materials and Methods. The 'uncontrolled' rates of choline oxidation during the aging study (Table II) were used to generate this curve. Both ethanol (○) and control (●) rates were used.

oxidation (35.2 nmol O_2/min per mg) which is consistent with the V results obtained in the dehydrogenase assay (Table I).

The decrease in mitochondrial ATP might be related to changes in the various mitochondrial ATPases. The effects of chronic ethanol ingestion on mitochondrial ATPase activity were determined in the presence and absence of exogenously added Mg^{2+} (Table III). When exogenously added Mg^{2+} was omitted from the incubation mixture, the intrinsic ATPase was found to be increased almost 3-fold in the mitochondria isolated from the ethanol-treated rats compared to controls (29.7 nmol/10 min per mg compared to 11.9 nmol/10 min per mg, respectively). In addition, both the Ca^{2+}-stimulated and dinitrophenol-stimulated ATPases were increased almost 2-fold as a result of chronic ethanol treatment (49.25 compared to 27.16 nmol/10 min per mg and 107.28

TABLE III

THE EFFECT OF CHRONIC ETHANOL INGESTION ON DIFFERENT ATPase ACTIVITIES IN RAT LIVER MITOCHRONDRIA

The numbers in parentheses represent the number of animals which were maintained on the liquid diet for 60 days. Each value represents the mean ± S.D. All ethanol values were different from controls at $P < 0.001$ when a paired t statistical test was used. The reaction mixture was assayed at 23°C and is described in Materials and Methods. It is essentially that of Gear [14]. The animals were fed ad libitum during the last 24 h prior to killing.

	Fed animals (nmol/P_i/10 min per mg)	
	Control (15)	Ethanol (16)
Intrinsic	11.9 ± 1.0	29.7 ± 5.9
Ca^{2+}	27.2 ± 2.8	49.3 ± 5.9
Dinitrophenol	53.1 ± 2.7	107.3 ± 5.3
Mg^{2+} *	23.3 ± 0.9	26.9 ± 1.9
Ca^{2+}/Mg^{2+} *	185.6 ± 4.5	173.2 ± 6.2
Dinitrophenol/Mg^{2+}	421.8 ± 9.9	393.3 ± 6.6

* Mg^{2+} was added to give a final concentration of 7.5 mM.

compared to 53.12 nmol/10 min per mg, respectively). Thus, these studies strongly suggest that chronic ethanol ingestion results in increases in ATPase activity in liver mitochondria. This is in agreement with Israel and Kuriyama [17] and results from Gordon's laboratory [18—20]. When Mg^{2+} was added to the incubations (7.5 mM final concentration), a small but significant increase in ATPase activity was noted in the mitochondria from the livers of the ethanol-treated animals. When this amount of Mg^{2+} was present, both the Ca^{2+}-stimulated and dinitrophenol-stimulated ATPase activities were slightly decreased in the mitochondria from the ethanol-treated animals. The results obtained when Mg^{2+} was added to incubation mixture corroborates other reports which suggest that ethanol ingestion has no effect on mitochondrial ATPase [21—23]. From these latter studies, it was concluded that by increasing the concentrations of Mg^{2+} in the reaction mixture, little or no differences could be demonstrated between controls and the ethanol-treated.

The fact that the intrinsic ATPase was increased in the mitochondria from the ethanol-treated animals and that the addition of 7.5 mM Mg^{2+} resulted in an increase in ATPase activity in the controls without affecting that from the ethanol-treated animals would seem to suggest that increased levels of Mg^{2+} could contribute to the increases in ATPase activity observed in the ethanol-treated animals. Therefore, the Mg^{2+} concentration of the isolated mitochondria was determined. It was found that Mg^{2+} concentration had increased from 21.52 ± 0.56 nmol/mg protein in the controls to 27.06 ± 0.43 nmol/mg protein in the ethanol-treated. This increase of 26% was significant at the $P < 0.001$ level of confidence. Thus, it is concluded that the increased mitochondrial Mg^{2+} levels in the ethanol-treated animals could contribute significantly to the increase observed in the mitochondrial ATPase (Table III).

It has been shown that fatty acids stimulated the Mg^{2+}-stimulated ATPase

TABLE IV

THE EFFECT OF CHRONIC ETHANOL INGESTION ON DIFFERENT ATPase ACTIVITIES IN RAT LIVER MITOCHRONDRIA

The numbers in parentheses represent the number of animals which were maintained on the liquid diet for 60 days. Each value represents the mean ± S.D. The reaction mixture was assayed at 23°C and is described in Materials and Methods. It is essentially that of Gear [14]. The animals were starved during the last 24 h prior to killing.

	Starved 24 h (nmol P_i/10 min per mg)	
	Control (5)	Ethanol (5)
Intrinsic	23.7 ± 0.5	49.6 ± 0.5 **
Ca^{2+}	52.4 ± 0.6	84.8 ± 2.7 **
Dinitrophenol	103.4 ± 3.4	196.9 ± 3.3 **
Mg^{2+} *	34.7 ± 0.8	35.7 ± 4.2
Ca^{2+}/Mg^{2+} *	181.8 ± 1.3	178.0 ± 3.6
Dinitrophenol/Mg^{2+}	426.1 ± 2.8	386.0 ± 4.8 **

* Mg^{2+} was added to give a final concentration of 7.5 mM.

** These ethanol values were different from controls at $P < 0.001$ when a paired t statistical test was used.

[24,25]; therefore, an additional study was conducted in an attempt to corroborate these observations and to expand the observations made in Table III. To insure that fatty acids were mobilized [26], the animals were fasted for 24 h before killing. The data in Table IV show that the intrinsic, Ca^{2+}-stimulated, dinitrophenol-stimulated and Mg^{2+}-stimulated ATPases were increased when compared to the data presented for the fed animals (Table III). However, it is important to note that ethanol-ingestion still resulted in an increased ATPase activity in the intrinsic, Ca^{2+}-stimulated and the dinitrophenol-stimulated ATPase. The addition of 7.5 mM Mg^{2+} to the intrinsic ATPase increased the activity found in the control and decreased the activity found in the ethanol-treated mitochondria such that there was no difference between the two mitochondrial preparations. It is concluded from these experiments that increased mobilization of fatty acids exerts a stimulating effect on mitochondrial ATPase; however, the effect of ethanol ingestion is still largely attributable to increased mitochondrial Mg^{2+} concentrations.

Consistent with the increase in ATPase, the efficiency of oxidative phosphorylation was decreased. The respiratory control ratio for β-hydroxybutyrate was 10.65 ± 1.18 with the control mitochondria and 7.81 ± 0.70 with the experimental mitochondria, and the ADP/O ratios were 2.83 ± 0.01 and 2.39 ± 0.01, respectively. These values represent the mean ±S.E.M. from four animals. When succinate was used as the substrate, the respiratory control ratio was 10.01 ± 0.4 in the control and 6.77 ± 0.31 in the ethanol-treated, and the ADP/0 ratios were 1.85 ± 0.03 and 1.64 ± 0.02, respectively. In all cases, the values from the ethanol-treated animals were statistically different from the controls at $P < 0.05$ or better. The ADP/0 ratios were calculated from the oxygraph tracings used to obtain the respiratory control ratios. Because Mg^{2+} was a component of the reaction mixture for the respiratory control determinations and because minimal changes were observed in the ATPase in the presence of Mg^{2+} (Tables III and IV), the ADP/0 ratios may not reflect directly the ATPase changes observed in Table III or IV in the absence of Mg^{2+}.

Discussion

De Ridder and van Dam [7] determined that the K_m for isolated choline dehydrogenase was 0.7 mM and that V was 35.0 nmol/min per mg. A comparison of these results with the data shown in Table I establishes the reliability of the polarographic and spectrophotometric assays. The studies of de Ridder and van Dam [27,7] utilized a lengthy procedure for the preparation of mitochondrial particles in which choline dehydrogenase had been separated from betaine aldehyde dehydrogenase. The assay of choline dehydrogenase as described by Singer [4] utilized a spectral measurement of the oxidation of dichlorophenol-indophenol coupled to phenazine methosulfate. Singer commented that pretreatment of fresh mitochondria with phospholipase A would assure full penetration of both choline and phenazine methosulfate [4]; however, it was observed (Fig. 1) that the addition of 1 mM $CaCl_2$ was sufficient to allow minimal activity in fresh liver mitochondria. The mitochondrial preparation used by de Ridder and van Dam [27,7] also depended on the intactness of the link between the dehydrogenase and the electron transport system. How-

ever, in the present report, the activity of choline dehydrogenase has been determined independent of electron transport activity. The convenience of these assays lies in the fact that freshly isolated mitochondria may be used by merely including 1 mM $CaCl_2$ in the reaction. The simplest interpretation of this effect of $CaCl_2$ is in terms of its ability to destroy the permeability barrier of mitochondria [28] and, thereby, to permit free penetration of the reactants. Because the uptake of choline was not stimulated by Ca^{2+}, the reactant with the limiting rate of penetration was phenazine methosulfate. The fact that phenazine methosulfate penetration is rate limiting was further supported by experiments involving the use of deoxycholate, freezing and thawing, and sonication. All of these treatments stimulated the rate of the choline-phenazine methosulfate reaction.

The particular type of double reciprocal plots generated from the kinetic analyses suggests that a ping-pong kinetic mechanism exists for the choline-phenazine methosulfate reaction. This is not usual for a mitochondrial dehydrogenase linked to the electron transport system. A ping-pong mechanism has been found to exist for the succinate-phenazine methosulfate reaction in certain types of fungi [29].

Use of the convenient and reliable spectrophotometric or polarographic assays has determined that the chronic ingestion of ethanol has no effect on the enzyme choline dehydrogenase. Neither the maximum activity of the enzyme nor the K_m for choline was altered. In contrast to these studies on the kinetic constants of this dehydrogenase, chronic ethanol ingestion had a marked effect on the total choline oxidase system (Table I). Polarographic studies on the oxidase system revealed that ethanol feeding increased the rate of choline oxidation and decreased the K_m for choline, and these effects of ethanol returned to normal after ethanol withdrawal. The fact that choline dehydrogenase was not altered, whereas, the choline oxidase system was, suggested that the ingestion of ethanol may have altered the control of choline oxidation at the level of the electron transport chain. This is somewhat in agreement with published work which suggests that ethanol ingestion has a selective effect on the mitochondrial electron transport system [30].

The fact that the presence of semicarbazide caused less than a 5% decrease in V of each assay was anticipated. Kagawa et al. [5] showed that the 'uncontrolled' phase of choline oxidation was more than 90% due to choline dehydrogenase activity. Even though the activity of betaine aldehyde dehydrogenase could be determined by difference (±semicarbazide), it was necessary to compare the oxidation rates of this substrate in mitochondria from control and ethanol-treated animals. Because other researchers have reported that ethanol metabolism partially uncouples site I of the mitochondrial electron transport system [30], it seemed possible that an increase in betaine aldehyde dehydrogenase activity could account for increased rates of choline oxidation. However, no difference in rates of betaine aldehyde oxidation was observed. Furthermore, the presence of $CaCl_2$ caused a 10-fold decrease in the activity of betaine aldehyde dehydrogenase in both the control and ethanol-treated mitochondria. Therefore, the increase in the 'uncontrolled' rate of choline oxidation as a result of chronic ethanol ingestion was due to alterations in other controls over this enzyme.

It has been reported that adenine nucleotides, especially ATP, exert control on choline oxidation [5—7]; however, different concentrations of ATP, ADP, and mixtures of both had no effect on choline dehydrogenase in our experiments. These results were in contrast to those of de Ridder and van Dam [7], who showed that ATP additions increased the K_m for choline and decreased the maximum activity in their assay for choline oxidation. However, in their studies [7], submitochondrial particles were used in which choline dehydrogenase had been separated from betaine aldehyde dehydrogenase but not from the electron transport system. Their results reflected the effect of adenine nucleotides on the oxidation of only choline. The different effects of adenine nucleotides on choline dehydrogenase activity compared to the reported effects on the choline oxidase system [5] suggest that adenine nucleotides exert their effects on the electron transport system rather than directly on choline dehydrogenase. Wilken et al. [6] further have attempted to isolate the location of the ATP and ADP effects. Using mitochondria inhibited with antimycin A, they measured the oxidation of cytochrome *c* and found that ADP and ATP inhibited cytochrome *c* oxidation. They suggested that the site of adenine nucleotide control over choline oxidation was somewhere prior to cytochrome *c* reduction. This data coupled with the data presented in this report would suggest that the inhibitory site lies between choline dehydrogenase and cytochrome *c* reductase.

Finally, the data from Table III suggest that increased ATPase activity may represent a persistent lesion in rat liver mitochondria as a result of chronic ethanol ingestion. The increased ATPase activity would seem to be a consequence of increased intramitochondrial levels of Mg^{2+}. The increase in intrinsic, Ca^{2+}-stimulated and uncoupler-stimulated ATPase activity as a result of chronic exposure to dietary ethanol is in sharp contrast to published reports [21—23,31]; however, the experiment in which 7.5 mM Mg^{2+} was added to the incubation medium is in agreement with these studies. Thus, we conclude that Mg^{2+} played an important role in regulating the mitochondrial ATPases. Further, Gordon's data [20] could be interpreted in two ways depending on which control is used. If the low fat control was used, then there was a 40% increase, and if the high fat control was used, then there was no difference. Cederbaum et al. [31] have found a slight decrease in the intrinsic, Ca^{2+}-stimulated and carboxyl cyanide *m*-chlorophenylhydrazone-stimulated ATPase activities as a result of chronic ethanol feeding with a high fat diet. The contrast between the results presented here and other published data [20—22,31] could be explained by the difference in the level of fat present in the diet. Further, the rates of ATPase activity reported by Cederbaum et al. [32] and Gordon [20] were 100-fold higher than those presented in Table IV. The ATPase activities reported here are more in line with other published work [14,32].

Cederbaum et al. [30] used EDTA in the homogenization medium, whereas in this report EGTA was used in the homogenization procedure. EDTA is a stronger chelating agent than EGTA for Mg^{2+} [33,34], which suggests that Cederbaum et al. [30] may simply have removed excessive amounts of Mg^{2+} from the mitochondria, thereby masking the effects of chronic ethanol ingestion. In fact, chronic ethanol-treated rats have been found to have mitochondrial Mg^{2+} contents similar to controls when livers were homogenized in an

EDTA-containing buffer [35]. Others have also observed that ethanol ingestion does not alter mitochondrial ATPase activity [21,36]. Both groups used EDTA in preparing the mitochondria, and both groups observed no alteration in respiratory control after ethanol ingestion. Hosein et al. [23] used a low fat diet (5%) and Videla et al. [21] used a medium fat diet (10%). Their techniques used to prepare the mitochondria, i.e. use of EDTA, could have contributed to their findings; however, they both measured the Mg^{2+}-stimulated ATPases. Our measurement of these ATPases also showed that there was no difference after ethanol ingestion. And as the data in Tables III and IV show, the presence of exogenously added Mg^{2+} masks the effects of ethanol. Therefore, if ethanol ingestion increased the mitochondrial ATPases, then decreased levels of mitochondrial ATP should have been observed as shown in Table II. These data are consistent with other data reported for whole liver [8,9]. Further, the decreased level of mitochondrial ATP could account for the increase in the 'controlled' rate of choline oxidation, and it may well explain the reduced lag time between the 'controlled' and 'uncontrolled' phase of choline oxidation. The curve generated by plotting the ATP content versus the 'uncontrolled' rate of choline oxidation suggests that 'uncontrolled' rates of choline oxidation could be used to determine mitochondrial levels of ATP.

The increase in ATPase activity, the decreased levels of ATP, the accelerated leakage of adenine nucleotides, and the greater susceptibility to the effects of aging of the mitochondria isolated from the ethanol-treated animals appear to suggest that these mitochondria do not exhibit the same structural integrity or intactness as control mitochondria. Therefore, these results may reflect a greater susceptibility of mitochondria from the ethanol-treated rats to damage during isolation. They also may reflect the structural changes in the fatty acid composition [3,36,37] as well as the phospholipid concentration [37,38] of mitochondria from livers of ethanol-treated rats. We [3,37], as well as others [36,39], have observed chronic ethanol ingestion to decrease the mitochondrial arachidonate/linoleate (20 : 4/18 : 2) ratio. Correlated with this change in fatty acid composition was a decrease in respiratory control for several mitochondrial substrates [3,38]. Using heart mitochondria from animals fed different lipids, Hsu and Kummerow [40] also have observed alterations in respiratory control with alterations in fatty acid composition. The 20 : 4/18 : 2 ratio did not correlate directly with the change in respiratory control, but it can still be said that the changes in fatty acid composition (which may be related to structural integrity) correlate with mitochondrial function in terms of substrate oxidation.

Unpublished work from this laboratory has indicated that an increase in liver free fatty acids may be responsible for altered choline metabolism during the early time course (5 days) effects of ethanol ingestion. A low fat diet containing ethanol does not give rise to the development of a fatty liver [41]. Therefore, the influx of dietary free fatty acids into the liver will be minimal under the conditions of our experiments. Further, the chronic nature of ethanol feeding does not promote fatty acid mobilization from adipose tissue [42]. Therefore, chronic ethanol ingestion results in an increased oxidation of choline by inducing an alteration in mitochondrial metabolism of adenine nucleotides, specifically ATP. Because both succinate and β-hydroxybutyrate

oxidation rates were decreased while choline oxidation was increased, the effect of ethanol ingestion most probably exists prior to the electron transport chain.

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