Biochemical Pharmacology, Vol. 27, pp. 2337–2342.
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0006-2952/78/1001-2337$02.00/0

EFFECTS OF CHRONIC ETHANOL TREATMENT UPON RAT LIVER MITOCHONDRIA*

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(*Received* 13 *May* 1977; *accepted* 26 *September* 1977)

Abstract—Liver mitochondria of male Sprague–Dawley rats chronically treated with ethanol showed a similar depression (40–50 per cent) of oxygen uptake under state 4, state 3 and DNP-uncoupled conditions with succinate as substrate. Using β-hydroxybutyrate as the substrate, respiratory rates in states 4 and 3 were depressed, but the rate under DNP-uncoupled conditions was not. ADP: O ratios were depressed by 16 per cent with pyruvate but not with succinate. Upon closer examination of the electron transport chain in freeze-thawed mitochondria, several heretofore undetected defects were noted. These include similar depressions (41–46 per cent) in the oxidations of NADH, β-hydroxybutyrate, malate or glutamate with cytochrome *c* as electron acceptor. These results suggest that defects do not occur at the level of substrate dehydrogenation but between NADH and cytochrome *c*. Other defects in the electron transport chain were observed: a 30 per cent decrease in NADH:ferricyanide reductase activity; a 38 per cent decrease in ubiquinone content and a 68 per cent decrease in succinate:cytochrome *c* reductase activity. However, the last result may not be attributed to effects upon succinic dehydrogenase *per se*, because succinate:DCIP reductase activity was unaltered. A 59 per cent depression of cytochrome *c* oxidase activity was also noted and must reflect a 44 per cent decrease in cytochrome aa_3 content, since K_m and V_{max} values were identical for control and ethanol-treated animals.

Chronic ethanol feeding has been shown to result in increased levels of mitochondrial lipid and a decreased rate of β-oxidation of fatty acids[1–4]. An increased rate of ethanol metabolism by the liver has also been noted[5–9]. To account for these observations a shift toward preferential metabolism of ethanol has been proposed[3]. The overall rate of ethanol metabolism may be limited by the ability of mitochondria to oxidize the cytoplasmic NADH produced by oxidation of ethanol[8–11]. However, it has been shown that activities of shuttles which transport reducing equivalents into the mitochondria are unaltered by ethanol treatment[12, 13] and that mitochondrial, but not cytoplasmic, ratios of NAD^+:NADH are depressed[3]. A decreased ability of the mitochondria to metabolize NADH has been proposed[3]. Various other aberrations in mitochondrial function and content have been reported, including depressions in cytochrome aa_3 content and in cyto- and mitoribosomal protein synthesis. By initiating a study of the effects of chronic ethanol feeding on synthesis of mitochondrial proteins, we attempted to use some of the reported effects of ethanol to delineate a suitable interval for study of changes in protein synthetic capacity and/or character. As the study proceeded, we found that the actions of ethanol on liver mitochondria which we were observing were somewhat different in several respects from those previously reported by others. We seek to detail these differences.

*Supported in part by grants from the NC Alcoholism Research Authority (7506) and from the National Institutes of Health (AG00256).

†To be submitted to the Graduate School of this institution in partial fulfillment of the requirements for the Ph.D. degree.

MATERIALS AND METHODS

Diet. Male Sprague–Dawley rats, 300–400 g, obtained from Charles River Breeding Labs., Inc. (Wilmington, MA), were fed a nutritionally well-balanced liquid diet in which carbohydrate, protein and fat contributed 69.9, 23.3 and 4.6% of the total calories respectively. When using ethanol, it isocalorically replaced dextrose for 35.5% of the total calories (see Thompson and Reitz[14]). Rats were divided by weight into groups of three animals; two received dietary ethanol and the control was fed the preceding day's average caloric intake of the two animals receiving ethanol. Animals were gradually introduced to the ethanol diet by replacing 18% of the total calories with ethanol the first 3 days, 27% the next 4 days and 35.5% on day 8. The animals consumed a total of about 90 kcal/day; they were sacrificed after 40–60 days on the diet. Essentially identical rates of weight gain were sustained by animals on the control (1.33 ± 0.3 g/day) and ethanol (1.42 ± 0.1 g/day) diets.

Mitochondria. Mitochondria were prepared according to the method of Chappell and Hansford[15] by differential centrifugation, using a medium containing 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES (*N*-2-hydroxyethyl-N′-2-ethanesulfonic acid) and 0.5 mg/ml of BSA (bovine serum albumin), pH 7.4[16]. Final pellets were drained well and homogenized in 2–3 ml of the same medium without BSA, and the protein concentration was determined by the method of Lowry *et al.*[17]. Two dilutions of the mitochondrial suspension in the BSA-free isolation medium were then prepared and the protein concentration was redetermined; one suspension was used for polarographic work (50 mg/ml) and the other was used for all other determinations (20 mg/ml). When assaying enzymes, suspensions were frozen at −70° and thawed at room temperature just prior to use.

Polarographic recordings. Oxygen uptake was measured polarographically at 25° in a YSI 5331 oxygen probe (Yellow Springs, Inst. CO). The mitochondrial suspension (0.1 ml) was added to 2 ml of respiration medium containing 10 mM pyruvate (plus 1 mM L-malate), 10 mM β-hydroxybutyrate (BHB) or 5 mM succinate as outlined by Schnaitman and Greenawalt[16]. ADP:O and respiratory control ratios (RCR) were calculated according to Estabrook[18]. Other details are indicated in the text.

Enzyme assays. Freshly thawed mitochondria (20 μg protein) were used in all assays except where specifically noted. Triplicate spectrophotometric determinations were made at 25° and initiated by the addition of substrates. Measurements of cytochrome *c* reduction with various substrates were performed essentially as described by Sottocasa *et al.*[19], except that a concentration of 0.01 mM cytochrome *c* was used. The concentrations of substrates employed were as follows: NADH, 1 mM; glutamate, malate and β-hydroxybutyrate, 3 mM + 1 mM NAD^+; and succinate, 3 mM.

Cytochrome *c* oxidase activity was measured as described by Elliott *et al.*[20] using 0.009 mM cytochrome *c*, 0.05 per cent Tween-80, 0.5 mM EDTA and 50 mM phosphate, pH 6.0. Cytochrome *c* was reduced with sodium dithionite and treated with Bio-Rex RG 501-X8 mixed bed resin (Bio Rad Labs, Richmond, CA) to remove excess reductant, prior to use in the assay. Succinic dehydrogenase activity (succinic:DCIP reductase) was measured according to King[21] using 100 μg of mitochondrial protein, 8 mM succinate, 0.06 mM phenazine methosulfate (PMS), 0.02 mM of 2,6-dichlorophenol-indophenol (DCIP), 1 mM potassium cyanide and 50 mM phosphate, pH 7.5. NADH dehydrogenase activity [NADH:$K_3Fe(CN)_6$ reductase] was determined using 2 mM NADH, 1 mM $K_3Fe(CN)_6$, antimycin A_3 (0.625 μmole/g of mitochondrial protein) and 50 mM phosphate, pH 7.5 [22]. Antimycin A_3 (5 μl of a 2.5 μM solution in ethanol) was added to both the sample and reference cuvettes and preincubated 2 min with mitochondria and buffer, as suggested by Slater [23]. In succinic and NADH dehydrogenase assays, reference cuvettes were so constituted as to compensate for any nonenzymatic reduction of electron acceptors. Saturating levels of substrates were used in all assays, and rates were linear during the period of measurement.

Estimation of cytochromes. Freshly thawed mitochondrial suspension was mixed with an equal volume of 4 per cent Triton X-100 and 0.4 M potassium phosphate, pH 7. After centrifugation at 5000 *g* for 15 min, the supernatant fraction was divided between two cuvettes (1 cm light path) and the contents of one cuvette were reduced for 20 min with a few crystals of sodium dithionite. Reduced minus oxidized difference spectra were recorded between 500 and 650 nm. Millimolar extinction coefficients for cytochrome aa_3 (605–630 nm) and cytochrome $c + c_1$ (550–540 nm) of 12 and 19.1, respectively, were used to calculate cytochrome concentrations as described by Wilson [24]. Cytochrome *c* was extracted and determined according to the procedure of Slack and Bursell [25] using 0.2 ml of the mitochondrial suspension. All extractions and determinations were made in duplicate.

Ubiquinone estimation. Ubiquinone was extracted by the method of Kroger and Klingenberg [26] and estimated according to Redfearn[27].

Chemicals. NADH, ADP, L-malate, succinate, pyruvate, D,L-β-hydroxybutyrate, DNP, DCIP, PMS, BSA (fraction V) and cytochrome *c* (type III, horse heart) were obtained from Sigma Chemical Co., St. Louis, MO. D-Mannitol, L-glutamate and Triton X-100 were obtained from Fisher Scientific Co., Pittsburgh, PA. Sodium dithionite and potassium ferricyanide were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. The following chemicals were obtained from the indicated sources: enzyme-grade sucrose (Mann Research Labs., Orangeburg, NY); HEPES (Calbiochem, La Jolla, CA); potassium borohydride (Metal Hydrides, Inc., Beverly, MA) and antimycin A_3 (Upjohn Co., Kalamazoo, MI).

RESULTS

Respiration and energy transduction. Mitochondria of ethanol-treated animals showed a decreased rate of oxygen uptake with succinate (Table 1). Oxygen uptake was depressed to a

Table 1. Effects of chronic ethanol treatment on respiration of isolated rat liver mitochondria*

Respiration state	Control	Ethanol-treated	Change (%)	P
Succinate				
State 4	31.6 ± 12.9 (4)	18.4 ± 3.1 (5)	−42	0.1 > P > 0.05
State 3	110.0 ± 28.0 (3)	63.7 ± 17.8 (5)	−42	<0.05
DNP	237.0 ± 99.7 (4)	120.0 ± 18.8 (4)	−49	<0.05
RCR	3.06 ± 0.64 (3)	3.61 ± 0.34 (5)	—	NS
β-Hydroxybutyrate				
State 4	11.3 ± 1.6 (3)	7.6 ± 2.2 (7)	−33	<0.05
State 3	49.0 ± 8.7 (3)	37.4 ± 4.3 (6)	−24	<0.05
DNP	51.9 ± 1.2 (2)	53.8 ± 10.9 (6)	—	NS
RCR	4.31 ± 1.54 (3)	4.76 ± 0.37 (6)	—	NS

*Results, except for respiratory control ratios (RCR), are expressed as natoms oxygen uptake/min/mg of protein (mean ± S. D.), with the number of animals indicated in parentheses. Oxygen uptake was measured polarographically. Duplicate incubations were at 25°, containing about 5 mg of mitochondrial protein and 10 mM β-hydroxybutyrate or 5 mM succinate as substrate, and other components as described in Materials and Methods. DNP and ADP, when used, were 227 and 93 μM respectively. NS = not significant.

similar extent in state 4 (42 per cent), state 3 (ADP energized, 42 per cent), and in the DNP-uncoupled (49 per cent) state. With β-hydroxybutyrate, respiration was also depressed in both states 4 and 3, though to lesser extents (33 and 24 per cent respectively). Surprisingly, there was no apparent effect of ethanol upon DNP-uncoupled oxygen uptake with β-hydroxybutyrate as substrate. When ADP:O ratios were measured (Table 2), no significant change was noted with succinate as substrate, but with pyruvate, a 16 per cent decrease in this parameter was observed. The fact that succinate oxidation was depressed to similar extents in all three respiratory states while ADP:O ratios were unchanged suggests that ethanol induced a lesion or lesions in the respiratory chain but not in the coupling apparatus of sites II or III. On the other hand, the inhibitions of β-hydroxybutyrate oxidation must be attributed to effects of ethanol on the coupling apparatus because the ADP:O ratio with an NAD^+-linked substrate was depressed by 16 per cent (Table 2), roughly half of the contribution of site I to phosphorylation. Thus, the lack of effect of ethanol upon DNP-uncoupled oxidation of β-hydroxybutyrate seems to indicate that the defects in coupled respiration (Table 1) stem from an action on coupling site I. Despite the effect of chronic ethanol treatment on site I phosphorylation, no significant change was noted in the respiratory control ratio with β-hydroxybutyrate (Table 1). This type of uncoupling was previously noted by Videla *et al.*[9] in chronic ethanol feeding studies.

Cytochrome and ubiquinone contents. In their studies, Rubin *et al.* [28] noted a 20 per cent decrease in cytochrome aa_3 content in liver mitochondria after chronic ethanol feeding. In our experiments, we have confirmed that this change occurs but we have found that it is actually more profound than reported by Rubin *et al.*[28]. As seen in Table 3, chronic ethanol treatment resulted in a 44 per cent decrease in the content of cytochrome aa_3/mg of protein, while cytochrome $c + c_1$ and extractable cytochrome *c per se* were unaltered. The ratio of cytochrome $aa_3/c + c_1$ was depressed 38 per cent from the control value. The normal value found by Williams [29] for the ratio of cytochrome $aa_3/c + c_1$ is 0.7 in liver mitochondria of adult male Sprague–Dawley rats; a value of 0.67 was obtained for rats fed the control liquid diet (Table 3).

Consistent with the foregoing, it was found that the specific activity of cytochrome *c* oxidase was dramatically affected by ethanol treatment. The cytochrome *c* oxidase activity of lysed mitochondria was depressed 59 per cent by chronic ethanol feeding (Fig. 1A). This decrease must be attributed to the 44 per cent decrease in cytochrome aa_3 content in such mitochondria because the kinetic constants of the enzyme (V_{max} and K_m) were found to be identical in mitochondria of control and ethanol-treated animals. Proof of this point is to be seen in Fig. 1B. Cederbaum *et al.*[13] have also reported a decrease in the specific activity of cytochrome *c* oxidase in liver mitochondria after chronic ethanol administration.

In pursuing the study of additional changes induced by ethanol feeding, mitochondrial contents of ubiquinone were determined. As shown in Table 3, we found that the content of this key soluble component of electron transport is also depressed substantially (38 per cent). This was an unexpected and, to our knowledge, a heretofore undetermined consequence of chronic ethanol

Table 2. Effects of chronic ethanol treatment on ADP:O ratios of isolated rat liver mitochondria*

Substrate	Control	Ethanol-treated	Change (%)	P
Succinate	1.53 ± 0.4 (4)	1.49 ± 0.3 (6)	—	NS
Pyruvate + malate	2.99 ± 0.4 (4)	2.51 ± 0.4 (5)	−16	0.1 > P > 0.05

*Results are expressed as means ±S. D., with the number of animals indicated in parentheses. Duplicate incubations were performed as detailed in Table 1 and in Materials and Methods. Where indicated, pyruvate and malate were present at 10 and 1 mM concentrations respectively. Two hundred nmoles ADP were added to determine ADP:O ratios.

Table 3. Cytochrome and ubiquinone content of liver mitochondria of control and ethanol-treated rats*

Parameter	Control	Ethanol-treated	Change (%)	P
Cytochrome aa_3	0.21 ± 0.06 (7)	0.12 ± 0.04 (11)	−44	<0.01
Cytochrome $c + c_1$	0.32 ± 0.07 (7)	0.03 ± 0.07 (11)	—	NS
Cytochrome *c*	0.15 ± 0.07 (3)	0.15 ± 0.04 (6)	0	NS
Ubiquinone	2.4 ± 1.0 (6)	1.5 ± 0.45 (9)	−38	<0.05
Ratio of $aa_3/c + c_1$	0.67 ± 0.09 (7)	0.41 ± 0.15 (11)	−38	<0.001

* Results are expressed as nmoles/mg of mitochondrial protein (means ±S. D.); the number of animals used is indicated in parentheses. Duplicate determinations of cytochrome and ubiquinone levels were as described in Materials and Methods. NS = not significant.

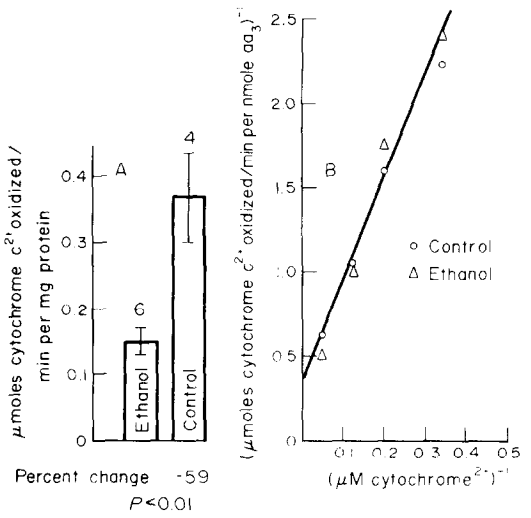

Fig. 1. Influence of chronic ethanol treatment on the specific activity and kinetic constants of rat liver cytochrome *c* oxidase. Panel A: bars represent the mean ±S. D., with the number of animals indicated at top. Triplicate assays were performed as indicated in Materials and Methods. Panel B: specific activity of cytochrome *c* oxidase as a function of cytochrome c^{2+} concentration. V_{max} is 2.94 μmoles cytochrome c^{2+} oxidized/min/nmole of heme aa_3; K_m (apparent) is 20 μM.

feeding. However, it is a change which is consistent with and helps to explain the aberrations of mitochondrial function induced by ethanol.

Isolated segments of electron transport. The observed decreases in ubiquinone and cytochrome aa_3 content and the effects upon oxygen uptake noted above led us to explore further the character of electron transport activity in the absence of phosphorylation. To obviate permeability problems, freeze-thawed mitochondria were used to measure the activities of various segments of the respiratory chain [28]. Specific activities of NADH: cytochrome *c*, β-hydroxybutyrate: cytochrome *c*, malate: cytochrome *c* and glutamate: cytochrome *c* reductases were each depressed to a similar extent (41–46 per cent), as shown in Fig. 2. These findings suggest that depressions in the rate of cytochrome *c* reduction stem from defects in the respiratory chain between NADH and cytochrome *c* and that defects do not occur at the level of substrate oxidations (e.g. β-hydroxybutyrate, malate or glutamate dehydrogenases).

To determine if ethanol had caused other defects in the respiratory chain between NADH and cytochrome *c*, we examined the activity of NADH dehydrogenase, as manifest by NADH: ferricyanide reductase activity. The specific activity of this enzyme was depressed by 30 per cent (Fig. 2) and may, in part, together with the 38 per cent decrease in ubiquinone level, account for the observed 41–46 per cent depression in the capacities for reduction of cytochrome *c* by NADH or the NAD^+-linked substrates. It should be emphasized that sufficient antimycin A_3 was used in the NADH: ferricyanide reductase assays so that the possibility of reduction of ferricyanide at other sites of electron transport was avoided [22].

The effects of chronic ethanol treatment were also assessed upon succinate oxidation by liver mitochondria. As can be seen in Fig. 2, succinate: cytochrome *c* reductase was depressed 68 per cent in mitochondria of ethanol-treated rats. Interestingly, this decrease could not be attributed to a diminished activity of succinic dehydrogenase *per se* because succinic: DCIP reductase activity was unaltered from control values in these same mitochondrial preparations. Other possibilities for this anomaly are considered in the Discussion.

DISCUSSION

These findings lead us to conclude that ethanol, when administered at the level of intake and for the length of time of our experiments, exerts its principal effects on the capacity of isolated mitochondria to oxidize substrates, through effects on both the electron transport chain and phosphorylation at coupling site I. Results of experiments which indicate damage to coupling site I, as an effect of chronic ethanol treatment, are supported by findings of previous investigators [9, 30]. Our

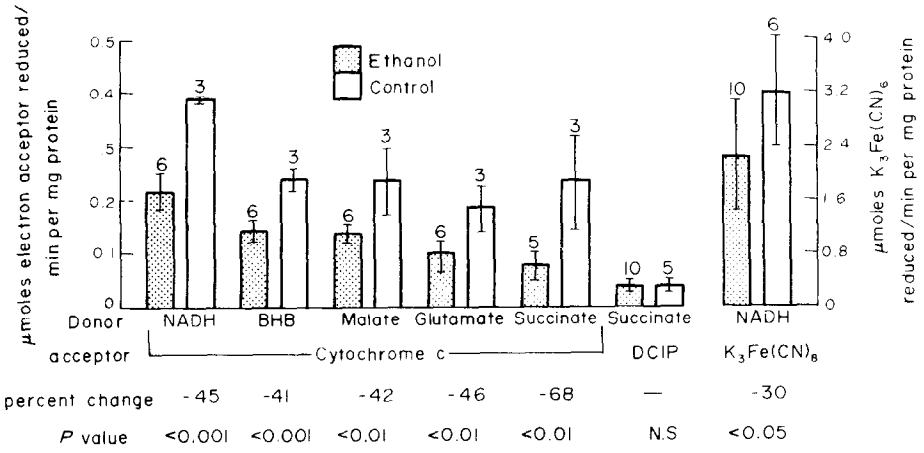

Fig. 2. Effects of chronic ethanol administration on the electron transport activity of lysed mitochondria. Conditions: triplicate enzyme assays were performed as indicated in Materials and Methods. Bars indicate the mean ±S. D. with the number of animals indicated above.

experiments indicate that chronic ethanol ingestion causes a 16 per cent decrease in the ADP:O ratio of mitochondria oxidizing pyruvate. While not too impressive in itself, this figure indicates a partial uncoupling of site I, on the order of 50 per cent of its contribution to total phosphorylation. Videla *et al.* [9] have noted a similar decrease (12 per cent) in the ADP:O ratio using β-hydroxybutyrate, while the respiratory control ratio was unaltered.

Further confirmation of our results which suggest that the effects of ethanol are more concentrated on the electron transport chain comes from a recent report of Koch *et al.*[31]. They report that respiration, the ADP:O ratio and the respiratory control ratio with β-hydroxybutyrate as substrate were not significantly altered, while respiration with succinate was depressed by approximately 50 per cent.

On the other hand, Cederbaum *et al.* [30] reported a much greater extent of damage to the coupling apparatus of site I as a result of chronic ethanol treatment. They reported a 25–30 per cent decrease in the respiratory control ratio and a 40 per cent decrease in ADP:O ratios with all NAD^+-linked substrates tested, while these parameters were unaffected when succinate served as substrate. In other experiments, Cederbaum *et al.* [30] demonstrated a 3-fold greater depression of respiration with NAD^+-linked substrates in state 3 than in state 4, while, with succinate or ascorbate, the degree of depression was similar in either respiratory state. In contrast to both our findings as well as those of others [9, 30, 31], Gordon [3] found that chronic ethanol ingestion greatly depressed the ADP:O ratio obtained with succinate as substrate; this effect was attributed to a decreased activity of adenine nucleotide translocase.

As indicated above, Cederbaum *et al.* [30] found that state 4, 3 and DNP-uncoupled respiration rates on succinate were minimally depressed (15–20 per cent) in ethanol-treated animals, and concluded that, while there were defects in the electron transport chain, the predominant effects of ethanol were concentrated on the coupling apparatus of site I. On the other hand, the effects noted in our study, 40–50 per cent decreases in succinate oxidation in all three states, indicate much more severe damage to the electron transport chain.

Our experiments demonstrate several severe impairments in electron transport capacity. Impaired electron flow is observed under coupled conditions (states 3 and 4) in intact mitochondria as well as in freeze-thawed mitochondria. Depressed respiratory rates stem from a multiplicity of deleterious actions of ethanol on rat liver mitochondria. Two especially discrete effects were observed: the depression of content of both ubiquinone and cytochrome *c* oxidase. The decrease in ubiquinone levels has not been previously reported and must contribute to the substantial depression of cytochrome *c* reduction when measured with any substrate tested. The studies of cytochrome *c* oxidase confirm and extend previous findings[13]. Under the experimental conditions employed, the loss of cytochrome *c* oxidase is more extensive than previously reported. It should be noted at this point that we have not assessed whether total liver content of cytochrome *c* oxidase is depressed by ethanol. However, because chronic ethanol administration for a comparable time, at the same level of intake, has been reported to cause no change either in mitochondrial protein/g of liver or in yield of mitochondria [32], we interpret the observed decrease as a change in specific mitochondrial content. The results of Fig. 1 do indicate, however, that the enzyme which is present is of unaltered character. Recent results of this laboratory demonstrate a decreased rate of synthesis of cytochrome *c* oxidase as a result of chronic ethanol treatment (unpublished observations).

One surprising result was that the oxidation of NADH or of the NAD^+-linked substrates (with cytochrome *c* as acceptor) were equally affected, but to lesser extents than that of succinate (succinate:cytochrome *c* reductase), while the specific activity of NADH dehydrogenase but not of succinic dehydrogenase was depressed. Since both NAD^+- and FAD-linked substrates are believed to share a common pool of ubiquinone, the diminished levels of this respiratory component along cannot account for the greater effect of ethanol on succinate oxidation. One potential explanation for this may stem from the decrease in cytochrome *b* level in ethanol-treated animals which has been noted by Rubin *et al.* [28]. A number of studies[33–36] suggest that FAD- and NAD^+-linked substrates do not share at least one of possibly three distinct cytochrome *b* pools. A selective decrease in a particular form of cytochrome *b* which is unique to or more involved in the oxidation of succinate could well explain the much more severe depression of succinate:cytochrome *c* reductase activity as an effect of chronic ethanol treatment. Such an effect would explain both the greater depressions in succinate oxidation with cytochrome *c* as acceptor in freeze-thawed mitochondria and in succinate supported oxygen uptake in coupled mitochondria (states 3 and 4) as compared to that of the NAD^+-linked substrates.

In seeking to understand the discrepancies evident in the findings of different laboratories, a comparison of the character and length of administration of the ethanol-containing diet in various studies might prove of benefit. The diet we have employed in our studies is that of Thompson and Reitz [14], which is fed *ad lib.* It is identical in ethanol content to all the others [3, 9, 40, 31]. The diet of Koch *et al.* [31] has twice the lipid content (9% of total calories), 56 per cent of the protein content (13% of total calories) and a similar content of carbohydrate. The diet used by Videla *et al.* [9] was also low in fat (10%) and very similar to that used in this study with respect to protein and carbohydrate content. In contrast, previous investigators, who have observed more severe defects upon coupling, have fed diets of lesser protein and carbohydrate contents and vastly elevated levels (in comparison) of lipid content, containing as much as 36 per cent [30] and 40 per cent [3] of total calories as lipid. Decreases in

β-oxidation of fatty acids and increased levels of lipids in hepatocytes as a consequence of chronic ethanol ingestion are well documented [1–4]. Thus, it is not unreasonable to conclude that a greater level of lipid ingested in conjunction with ethanol might lead to a condition wherein liver mitochondria from such animals are either inherently more unstable or more susceptible to damage during isolation, as evidenced by low respiratory control ratio values [3].

An additional basis for different results might involve length of time of ingestion of the diet. Experimentation with the diets of high lipid content has involved intervals of 21–30 days [3, 30], a substantially shorter interval than that which we have employed. Thus, the greater depression of cytochrome *c* oxidase, the large decrease in ubiquinone and the increased susceptibility of the electron transport chain relative to the coupling apparatus may stem, at least in part, from more prolonged exposure to ethanol. By the same token we believe that the longer exposure to the intrinsic actions of ethanol *per se*, in the absence of the complications of excessive levels of dietary lipid, may actually afford a clearer picture of the deleterious actions of ethanol. This does not indicate that we choose to ignore the importance of the diet in determining the extent and character of the effects of chronic ethanol ingestion.

REFERENCES

1. E. R. Gordon and J. Lough, *Lab. Invest.* **26**, 154 (1972).
2. C. S. Lieber, in *Metabolic Changes Induced by Alcohol* (Eds G. A. Martini and C. Bode), p. 212. Springer, New York (1971).
3. E. R. Gordon, *J. biol. Chem.* **248**, 8271 (1973).
4. A. I. Cederbaum, C. S. Lieber, D. S. Beattie and E. Rubin, *J. biol. Chem.* **250**, 5122 (1975).
5. R. D. Hawkins, H. Kalant and J. M. Khanna, *Can. J. Physiol. Pharmac.* **44**, 241 (1966).
6. C. S. Lieber and L. M. DeCarli, *J. biol. Chem.* **245**, 2505 (1970).
7. F. Tobon and E. Mezey, *J. Lab. clin. Med.* **77**, 110 (1971).
8. L. Videla and Y. Israel, *Biochem. J.* **118**, 275 (1970).
9. L. Videla, J. Bernstein and Y. Israel, *Biochem. J.* **134**, 507 (1973).
10. M. Theorell and B. Chance, *Acta chem. scand.* **5**, 1127 (1951).
11. J. J. Vitale, D. M. Hegsted, H. McGrath, E. Grafla and M. Zamcheck, *J. biol. Chem.* **210**, 753 (1954).
12. A. I. Cederbaum, C. S. Lieber, D. S. Beattie and E. Rubin, *Biochem. biophys. Res. Commun.* **49**, 649 (1972).
13. A. I. Cederbaum, C. S. Lieber, A. Toth, D. S. Beattie and E. Rubin, *J. biol. Chem.* **248**, 4977 (1973).
14. J. A. Thompson and R. C. Reitz, *Ann. N.Y. Acad. Sci.* **273**, 194 (1976).
15. J. B. Chappell and R. G. Hansford, in *Subcellular Components-Preparation and Fractionation* (Ed. G. D. Birnie), pp. 77–92. Butterworths-University Park Press, London (1972).
16. C. Schnaitman and J. W. Greenawalt, *J. Cell Biol.* **38**, 158 (1968).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. R. W. Estabrook, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman), p. 41. Academic Press, New York (1967).
19. G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, *J. Cell Biol.* **32**, 415 (1967).
20. W. B. Elliott, J. P. Holbrook and R. Penniall, *Biochim. biophys. Acta* **251**, 277 (1971).
21. T. E. King, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman), p. 322. Academic Press, New York (1967).
22. G. Schatz and E. Racker, *J. biol. Chem.* **241**, 1429 (1966).
23. E. C. Slater, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman), p. 48. Academic Press, New York (1967).
24. D. F. Wilson, *Biochemistry* **8**, 2475 (1969).
25. E. Slack and E. Bursell, *Biochim. biophys. Acta* **256**, 287 (1972).
26. A. Kroger and M. Klingenberg, *Biochem. Z.* **344**, 317 (1966).
27. E. R. Redfearn, in *Methods in Enzymology* (Eds R. W. Estabrook and M. E. Pullman), p. 381. Academic Press, New York (1967).
28. E. Rubin, D. S. Beattie and C. S. Lieber, *Lab. Invest.* **23**, 620 (1970).
29. J. N. Williams, *Biochim. biophys. Acta* **162**, 175 (1968).
30. A. I. Cederbaum, C. S. Lieber and E. Rubin, *Arch. Biochem. Biophys.* **165**, 560 (1974).
31. O. R. Koch, C. D. Bedetti, M. Gamboni, G. A. Montero and A. O. Stoppani, *Expl Molec. Path.* **25**, 253 (1976).
32. A. I. Cederbaum and E. Rubin, *Fedn Proc.* **34**, 2045 (1975).
33. K. A. Davis, Y. Hatefi, K. L. Poff and W. L. Butler, *Biochem. biophys. Res. Commun.* **46**, 1984 (1972).
34. C. Yu, L. Yu and T. E. King, *Biochem. biophys. Res. Commun.* **66**, 1194 (1975).
35. L. Ernster, I. Lee, B. Norling and B. Presson, *Eur. J. Biochem.* **9**, 299 (1969).
36. W. Waino, in *The Mammalian Mitochondrial Respiratory Chain* (Eds B. Horecker, N. Kaplan, J. Marmur and H. A. Scheraga), p. 238. Academic Press, New York (1970).