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Effects of chronic ethanol consumption on the synthesis of polypeptides encoded by the hepatic mitochondrial genome

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Liver mitochondria from rats fed ethanol chronically demonstrate an impaired ability to incorporate [^{35}S]methionine into polypeptide products in vitro. This ethanol-induced effect on mitochondrial translation in vitro could not be attributed to significant differences in the methionine precursor pool sizes of ethanol and control mitochondria or to the acute effects of residual ethanol. The observed reduction of radiolabeled methionine incorporation into mitochondrial gene products of ethanol mitochondria in vitro reflects a decrease in the synthesis of all the mitochondrial gene products. However, the percentage of total radiolabel incorporated into each gene product is unaffected by ethanol, suggesting an ethanol-induced coordinate depression of mitochondrial protein synthesis. Moreover, SDS-PAGE and densitometry of submitochondrial particles from ethanol-fed and control rats demonstrated that the steady-state concentration of each of the mitochondrial gene products is decreased in ethanol-fed rats. This reduction of the steady-state concentration of the mitochondrial gene products may be related to the observed depressions of oxidative phosphorylation activities associated with hepatic mitochondria from ethanol-fed rats.

Introduction

Studies of the effects of chronic ethanol consumption on hepatic mitochondrial oxidative phosphorylation have demonstrated that the activity or content of specific segments of the oxidative phosphorylation system are adversely affected by ethanol, while other portions are apparently unaffected. Moreover, the altered components of the oxidative phosphorylation system all contain polypeptides which are encoded by the mitochondrial genome and are synthesized on mitochondrial ribosomes. It has been shown that the activities of NADH dehydrogenase [1–4], cytochrome oxidase [1,4–6] and the $F_0 \cdot F_1$-ATPase [3,7] are depressed in mitochondria from ethanol-fed rats. In addition, the content of cytochrome *b* [6,8] and heme *a* from cytochrome oxidase [1,6] have been shown to be reduced in ethanol mitochondria. In contrast, other components of the mitochondrial oxidative phosphorylation system including ubiquinone [6], succinate dehydrogenase [1,2], cytochromes $c + c_1$ [1,6] and the adenine nucleotide exchange protein [9] are unaffected by ethanol consumption. These components of the oxidative phosphorylation system are composed entirely of polypeptides synthesized in the cytoplasm and imported into the mitochondrion.

The above observations suggest that ethanol affects the processes involved in mitochondrial biogenesis. In the present study, mitochondrial protein synthesis was investigated in order to define more clearly the effects of chronic ethanol consumption on this particular process of mitochondrial biogenesis. Previous reports [10–12] have suggested that the rate of synthesis of mitochondrial gene products is depressed in ethanol mitochondria. The results presented in this paper demonstrate that chronic ethanol consumption adversely affects mitochondrial protein synthesis resulting in a reduction of the concentrations of the mitochondrial gene products in mitochondria of ethanol-fed rats. Evidence

Abbreviations: Ethanol mitochondria, mitochondria isolated from ethanol-fed rats; control mitochondria, mitochondria isolated from liquid-diet control rats; SMP, submitochondrial particles; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $F_0 \cdot F_1$-ATPase, the oligomycin-sensitive ATP synthase holoenzyme complex; AdNC, adenine nucleotide carrier; COI, COII and COIII, polypeptide subunits I–III of cytochrome oxidase; ATPase 6 and ATPase 8, polypeptide subunits 6 and 8 of the $F_0 \cdot F_1$-ATPase; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, polypeptide subunits 1–6 and subunit 4L of the NADH dehydrogenase complex; Cyt *b*, the polypeptide moiety of cytochrome *b*;

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is also presented which suggests strongly that the reduction of mitochondrial gene product concentration is related to the loss of oxidative phosphorylation activity observed with mitochondria from ethanol-fed rats.

Materials and Methods

Sprague-Dawley rats and several reagents were obtained from sources listed previously [13,14]. Preparations of rat liver cytochrome oxidase were the generous gift of Drs. Linda Gregory and Shelagh Ferguson-Miller, Department of Biochemistry, Michigan State University. Rabbit polyclonal antibody, directed against bovine cytochrome oxidase subunit II, was kindly provided by Drs. Yu-zhong Zhang and Rhoderick Capaldi, Institute of Molecular Biology, University of Oregon. L-[^{35}S]Methionine (> 1200 Ci/mmol) and a complete amino acid mixture minus methionine were obtained from the Amersham Corporation. Cycloheximide and chloramphenicol were from United States Biochemical Corporation. Bicine was from Aldrich Chemical Company, and Budget-Solv complete scintillation cocktail from Research Products International. Autoradiography enhancer and tissue solubilizer were purchased from New England Nuclear. Hydrogen peroxide, 4-chloro-1-naphthol and horseradish peroxidase conjugated goat anti-rabbit IgG were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

Male Sprague-Dawley rats initially weighing 150–250 g were fed for 31 days a nutritionally adequate diet [15] in which ethanol provided 36% of the total calories. Pair-fed control rats [15] received the same diet, but with maltose/dextrin isocalorically substituted for ethanol. Coupled mitochondria were prepared by differential centrifugation of liver homogenates essentially as described by Thayer and Rubin [5] with the addition of three washes of the mitochondrial pellet with 0.25 M sucrose/10 mM EDTA (pH 7.4) to eliminate contaminating cytoplasmic ribosomes. The number of washes employed in the preparation of these mitochondria minimized the possibility that any ethanol present in the liver of the rat at the time of sacrifice remained in the mitochondria upon isolation. Representative preparations of mitochondria from ethanol-fed and control rats gave respiratory control ratios of 2.6 ± 0.04 and 4.3 ± 0.12 ($n = 3$ pairs) respectively, with succinate as substrate. These values were comparable to values previously obtained with liver mitochondria prepared from ethanol-fed and control rats [9]. Submitochondrial particles were prepared from purified liver mitochondria by the method of Thayer and Rubin [5]. Protein concentrations were determined by the method of Lowry et al. [16].

Isolated rat liver mitochondria were radiolabeled with [^{35}S]methionine essentially as described by Hofmann and Hosein [10] utilizing modifications suggested by Mills et al. [17]. Mitochondria were incubated with 500 μCi [^{35}S]methionine at 30 °C in a mixture containing 50 mM Bicine, 90 mM KCl, 2 mM EDTA, 5 mM potassium phosphate, 10 mM ATP, 10 mM magnesium chloride, 5 mM phospho*enol*pyruvate, 50 μg/ml pyruvate kinase, 20 mM of each amino acid (minus methionine), 500 μg cycloheximide and 25 mg mitochondrial protein in a total volume of 5 ml. The pH of the reaction mixture was 7.5. The progress of the reaction was monitored by the periodic withdrawal of aliquots of the reaction mixture for scintillation counting. These aliquots were pipetted onto filter paper, precipitated on the paper with 5% (w/v) trichloroacetic acid and washed extensively in 5% (w/v) trichloroacetic acid prior to solubilization of the radiolabeled protein with tissue solubilizer. The washed and solubilized aliquots of mitochondria were monitored for radioactivity in Budget-Solv complete scintillation cocktail. The incorporation of radiolabel was inhibited by the inclusion of 100 μg/ml chloramphenicol in the labeling reaction.

The endogenous methionine pool sizes in isolated ethanol and control mitochondria were determined using a competition/dilution labeling assay. Under the conditions employed, this type of assay provides a measure of the accessible methionine pool which can be utilized for protein synthesis in the mitochondrion. In these studies mitochondria were labeled with 150 μCi [^{35}S]methionine, as described above, in a series of mixtures which included various additions of unlabeled methionine. Competition of the unlabeled methionine with the labeled amino acid for incorporation into elongating polypeptide chains reduced the amount of label incorporated proportionally with the amount of unlabeled methionine added. The percentage of maximum labeling was determined for several additions of unlabeled methionine in order to ascertain the amount of unlabeled methionine required to elicit a 50% reduction in the amount of label incorporated. The amount resulting in a 50% reduction in radiolabel incorporated is equal to the endogenous methionine pool size according to the relationship described by Faires and Boswell [18] for isotope dilution experiments.

SDS-PAGE was performed as described by Laemmli [19] utilizing slab gels containing either 15% (w/v) polyacrylamide or a linear gradient of 12.5–20% (w/v) polyacrylamide. Gels were stained with silver by the method of Wray et al. [20] or with 0.5% (w/v) Coomassie brilliant blue R-250 in isopropanol/acetic acid/water (3 : 1 : 6). Autoradiography was carried out at −70°C using Kodak X-Omat AR film. Fluorography was performed likewise following treatment of gels with autoradiography enhancer.

The rat liver mitochondrial gene products were identified through the specific radiolabeling of isolated rat liver mitochondria in the presence of cycloheximide, an inhibitor of cytoplasmic ribosomes. The resulting

[^{35}S]methionine labeled mitochondria were fractionated to SMP, separated by SDS-PAGE and used for autoradiography or fluorography. As illustrated in Fig. 4, fluorograms showed prominent radiolabeling of thirteen polypeptides in addition to other labeling products which have been observed previously [21]. The incorporation of [^{35}S]methionine into these thirteen polypeptides was inhibited by the inclusion of chloramphenicol in the reaction mixture. The susceptibility of the reaction to chloramphenicol, a specific inhibitor of mitochondrial ribosomes, verified that radiolabel was being incorporated into polypeptides of mitochondrial origin. The identities of the mitochondrially synthesized polypeptides were assigned based upon the relative migration positions of the homologous human mitochondrial gene products in SDS containing gels [22]. The mammalian mitochondrial DNAs [23–26] display a high degree of direct sequence homology, with most deviations in sequence between species representing either silent changes in the DNA or alterations producing amino acid substitutions with amino acids possessing similar properties [27]. Therefore, the mitochondrial gene products of human and rat are expected to possess similar physical characteristics and similar banding patterns on SDS-PAGE, which was our observation (Fig. 4).

The mitochondrially synthesized polypeptides were located in the Coomassie blue staining pattern of submitochondrial particles (separated by SDS-PAGE) through direct comparison of stained gels with their respective autoradiograms. The overall Coomassie blue banding pattern of SMP, as well as the relative position of individual protein bands in the gel, was important in determining which peaks derived from densitometric scans should be integrated. The assignments for cytochrome oxidase subunits I–III in the staining pattern of SMP were verified by secondary methods, as outlined below. Assignments for subunits 6 and 8 of the mitochondrial ATPase were further substantiated by a comparison with gel patterns obtained with the purified ATPase [7,14].

Quantitation of the Coomassie blue staining intensity of the mitochondrial gene products in stained gels was accomplished through automated computer integration of the appropriate peaks (those corresponding to mitochondrial gene products) obtained utilizing a Zenieh Model SL-TRFF scanning laser densitometer and an electrophoresis reporting program (ERIP-V3A) from Biomed Instruments. The values obtained through integration were normalized relative to the staining intensity of the adenine nucleotide carrier peak in order to correct for errors introduced through sample preparation or handling. The adenine nucleotide carrier is a cytoplasmically synthesized integral membrane protein [28] which has been shown to be unaffected by ethanol consumption [9]. Therefore, this polypeptide is expected to be present in normal amounts in mitochondrial membranes of ethanol-fed rats.

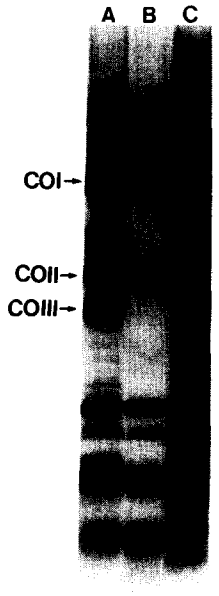

Fig. 1. Identification of cytochrome oxidase subunits I, II and III by SDS-PAGE. Protein preparations were separated on 15% (w/v) polyacrylamide slab gels and stained with silver as described in Methods. Lane A, cytochrome oxidase holoenzyme (5 μg); lane B, cytochrome oxidase lacking subunit III (5 μg); lane C, rat liver submitochondrial particles (15 μg). The migration positions of subunits I–III of cytochrome oxidase are indicated.

Since it was reported earlier [29] that cytochrome oxidase subunits I–III are present in mitochondria from ethanol-fed rats in normal concentrations, the identification of these mitochondrial gene products was more rigorously confirmed. The location of cytochrome oxidase subunit I was verified by coelectrophoresis of a rat liver cytochrome oxidase preparation with rat liver SMP. Subunit I is a high molecular weight component which appears as a broad band in the gel pattern (Fig. 1). Cytochrome oxidase subunit II was positively identified through cross reactivity to polyclonal IgG directed against the bovine cytochrome oxidase subunit II (data not shown), with immunoblots of electrophoretically transferred proteins to nitrocellulose being performed essentially as described by Towbin et al. [30]. Subunit III of cytochrome oxidase was identified through coelectrophoresis of rat liver SMP with rat liver cytochrome oxidase preparations which either contained or lacked subunit III (Fig. 1).

The values reported in the tables and figures are averages of multiple data acquisitions ± S.E. In figures where standard error bars are not shown the values were determined to be less than 15% of the means. Statistical analyses were performed using the paired *t*-test and the analysis of variance (ANOVA) with repeated measures, employing the Statview 512 + program from Brainpower.

Results

Determination of the time-course for incorporation of radiolabeled amino acid into mitochondrial gene products in vitro provides a measure of the relative abilities of isolated ethanol and control organelles to synthesize mitochondrially encoded polypeptides. The results shown in Fig. 2 demonstrate that there is a dramatic decrease in radiolabeled methionine incorporation into mitochondria isolated from ethanol-fed rats. The chloramphenicol sensitivity of this translation activity in vitro for both ethanol and control mitochondria indicated that radiolabel was being incorporated into mitochondrial gene products of each mitochondrial source. The ethanol-induced depression of mitochondrial translation activity in vitro, shown in Fig. 2, is of the order of 35–40% of control. The results shown in Fig. 2 were not attributable to differences in the energy state of the ethanol and control mitochondria since excess (10 mM) ATP and an ATP regenerating system (phospho*enol*pyruvate and pyruvate kinase) were included in the incubation medium. The exogenous ATP included in the reaction mixture should not have stimulated translational activity in control mitochondria more than in ethanol mitochondria, since the activity of the adenine nucleotide translocase is unaffected by chronic ethanol consumption [9]. Thus, the added ATP should have been equally available for both ethanol and control mitochondria. Even though mitochondria from ethanol-fed rats are deficient in their capacity to synthesize ATP [3,7,31], under the conditions employed ATP

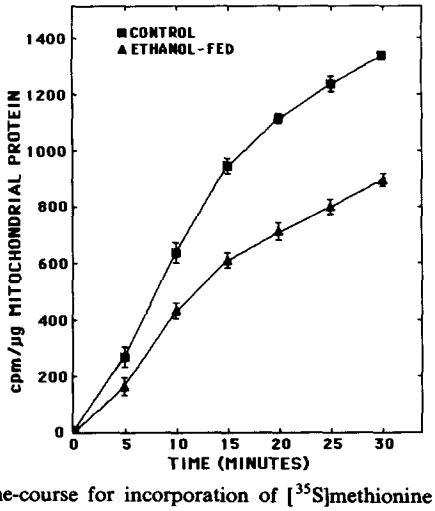

Fig. 2. Time-course for incorporation of [^{35}S]methionine into mitochondrial gene products of ethanol and control mitochondria in vitro. Each point represents the average chloramphenicol-sensitive acid-insoluble radioactivity incorporated per microgram of mitochondrial protein from seven animals ± S.E. Points without bars were values with S.E. encompassed within the height of the symbol. Differences between ethanol and control values at each time point were significant by the paired *t*-test, $P \leq 0.02$.

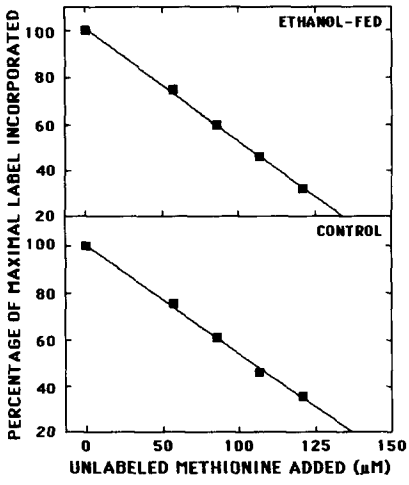

Fig. 3. Determination of the endogenous methionine pool sizes of ethanol and control mitochondria. Each point represents the average acid-insoluble radioactivity incorporated, expressed as the percentage of the maximum labeling, for each of seven mitochondrial preparations. The methionine pool sizes were calculated from these data utilizing the approach outlined in Materials and Methods.

should not have been limiting for mitochondrial protein synthesis [32].

To eliminate the possible acute effects of residual ethanol in the incubation mixture, mitochondria were washed extensively before radiolabeling experiments were performed. In addition, experiments were carried out to determine the effect of added ethanol on the incorporation of [^{35}S]methionine into proteins of mitochondria from liquid diet control animals. Ethanol was included in these experiments at a concentration of 33 mM, an amount equivalent to a blood ethanol level of 150 mg/dl. The incubation protocol is identical to that for the data in Fig. 2. The results of these studies showed no significant difference between the incorporation of radiolabel into mitochondrial gene products by control mitochondria in the presence or absence of 33 mM ethanol (data not shown). Thus, the decreased rate of methionine incorporation shown in Fig. 2 represents an effect of chronic ethanol consumption, rather than an effect of acute ethanol exposure on the isolated mitochondria. The endogenous free methionine pool sizes of ethanol and control mitochondria were determined in order to eliminate the possibility that the observed effects of chronic ethanol consumption on mitochondrial protein synthesis in vitro were due to differences in the specific activity of the methionine precursor pools. An analysis of the data shown in Fig. 3 revealed that the endogenous methionine pool sizes for ethanol and control mitochondria were 105 ± 2 μM and 109 ± 3 μM, respectively.

In order to investigate the quantitative nature of the ethanol-elicited alterations in mitochondrial gene product synthesis and steady-state abundance, the mitochondrially synthesized polypeptides had to be identified

correctly in SDS-PAGE staining patterns. This was accomplished by identifying those polypeptides in autoradiograms which (1) were radiolabeled in the presence of cycloheximide and (2) demonstrated dramatically decreased incorporation of [^{35}S]methionine when chloramphenicol was included in the incubation mixture. These mitochondrially synthesized polypeptides were located in the Coomassie blue staining pattern of SMP by direct comparison of autoradiograms and stained gels, with secondary verification of ATPase subunits 6 and 8 and cytochrome oxidase subunits I–III. Together, these observations and secondary verifications allowed the identification of the mitochondrial gene products with high confidence. Correct identification was confirmed by observing that there were uniform ethanol-elicited decreases in radiolabeling and staining intensities for the thirteen polypeptides selected. It is notable that there were no ethanol-related decreases in the staining intensities for the ($\alpha + \beta$) subunits of the ATP synthase and the adenine nucleotide carrier protein, nuclear gene products which are readily identified in gel patterns [7].

An analysis of the effect of chronic ethanol consumption on the radiolabeling of individual mitochondrial gene products in vitro was carried out. The purpose of this study was to determine if the effects of chronic ethanol consumption on mitochondrial protein synthesis in vitro reflect a decrease in the synthesis of all the mitochondrial gene products or some subset thereof. Mitochondria from ethanol and control rats were labeled with [^{35}S]methionine, fractionated to SMP, subjected to SDS-PAGE and fluorography. The resulting films (see Fig. 4) were analyzed by densitometry in order to determine the relative extent of radiolabeling of each mitochondrial gene product, taking the incorporation of radiolabel as a relative measure of the extent of polypeptide synthesis. The results presented in Fig. 5 demonstrate that chronic ethanol consumption results in a decrease in the rate of synthesis (as indicated by a decrease in the incorporation of [^{35}S]methionine) of each mitochondrial gene product. Identical studies were performed using radiolabeled unfractionated ethanol and control mitochondria. Autoradiograms from intact mitochondria also revealed an ethanol-related decrease in the incorporation of [^{35}S]methionine into mitochondrial gene products (data not shown).

An analysis was carried out of the radiolabeling of each mitochondrial gene product in vitro expressed as a percentage of total radiolabel incorporated into all mitochondrially synthesized polypeptides. The results demonstrated that while the total radioactivity incorporated/μg of mitochondrial protein is decreased in ethanol mitochondria (see Fig. 2), the radioactivity of each gene product expressed as a percentage of total radiolabel incorporated, is unaffected by ethanol consumption. Thus, while demonstrating an impaired

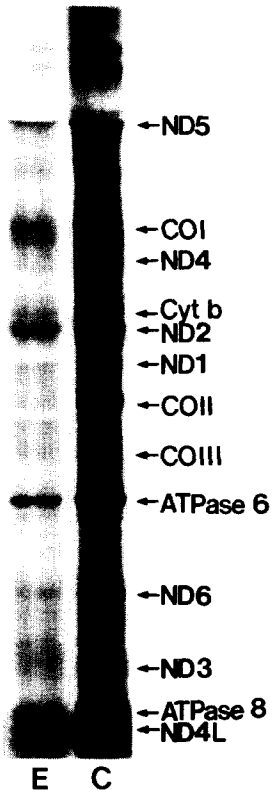

Fig. 4. Fluorography of [^{35}S]methionine labeled submitochondrial particles from ethanol and control rats. Identical amounts (20 μg) of SMP prepared from [^{35}S]methionine labeled ethanol and control mitochondria were separated on 12.5–20% (w/v) linear gradient polyacrylamide gels and used for fluorography. The migration positions of the mitochondrial gene products are designated using accepted abbreviations. The lane designated E contains SMP derived from ethanol mitochondria and that designated C contains SMP derived from control mitochondria.

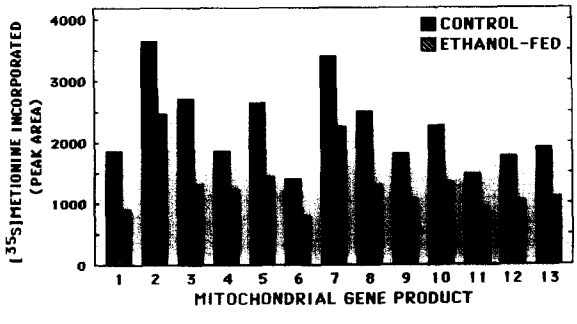

Fig. 5. Ethanol-induced depression of [^{35}S]methionine incorporation into mitochondrial gene products in vitro. SMP prepared from [^{35}S]methionine-labeled ethanol and control mitochondria were analyzed by SDS-PAGE (with identical protein loads) and fluorography. Fluorograms were densitometrically scanned to determine the relative extent of radiolabeling of each mitochondrial gene product. Each bar represents the average densitometrically determined radioactivity incorporated for a particular gene product from seven animals. The identity of each mitochondrial gene product (see Materials and Methods), designated here and in subsequent figures by number, are shown in Table I. The maximal standard error for these measurements was 6% of the mean value. The observed differences between ethanol and control values for each of the mitochondrial gene products were determined to be significant by statistical analysis utilizing the paired t-test ($P \leq 0.003$) and ANOVA ($P \leq 0.05$).

ability to synthesize polypeptides, ethanol mitochondria distribute the radiolabel into mitochondrial gene products in normal ratios.

Investigation of the steady-state quantities of the mitochondrial gene products in ethanol and control mitochondrial membranes was accomplished through SDS-PAGE and densitometry of SMP from ethanol and control rat livers. These studies showed ethanol-induced alterations in the protein composition of mitochondrial membranes. These changes were apparent when SMP from ethanol and control rats were analyzed by SDS-PAGE (Fig. 6A). The gel shown in Fig. 6A of SMP from an ethanol-fed and control rat, stained with silver, demonstrates clearly ethanol-elicited depressions in the levels of several mitochondrial gene products (COII, ND3, ATPase 8 and ND4L). While silver-stained gels demonstrated these alterations in the staining intensities of mitochondrial gene products more clearly, these ethanol-related changes could also be observed in gels of SMP stained with Coomassie blue. Gels stained with Coomassie were utilized for densitometric analyses in these studies since the light source in the densitometer available was more sensitive to the Coomassie blue stain. Fig. 7 illustrates complete densitometric scans for SMP from an ethanol-fed rat and its pair-fed control. Utilizing the gel scans for SMP from 12 pairs of animals and the integration procedures outlined in the Fig. 7 legend, it was demonstrated that chronic ethanol consumption results in a significant reduction of the steady-state concentrations of all the mitochondrial gene products in the inner membrane of ethanol-fed rats (Fig. 8).

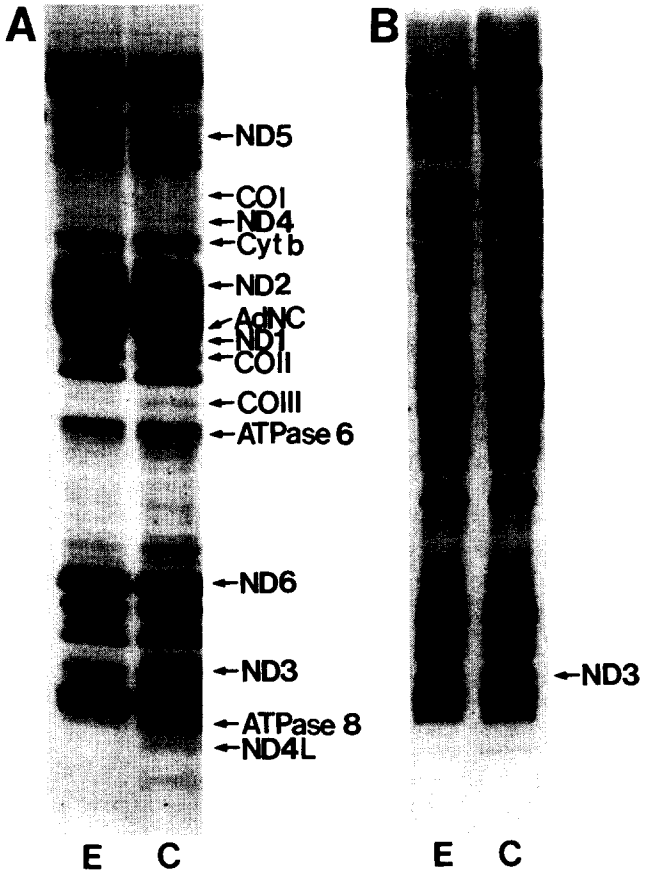

Fig. 6. SDS-PAGE of ethanol and control mitochondria and submitochondrial particles. In each case equivalent amounts of protein from ethanol and control preparations were utilized for the electrophoretic separation on 12.5–20% polyacrylamide gradient gels (as described in Materials and Methods). The identities of the rat liver mitochondrial gene products, assigned as described in Materials and Methods, are indicated employing accepted abbreviations. The adenine nucleotide carrier is indicated by AdNC. Lanes designated by E contain samples prepared from ethanol-fed rats, and lanes designated by C are from pair-fed control rats. (A) Submitochondrial particle preparations (15 μg) from ethanol-fed and control rats, stained with silver. Polypeptide bands corresponding to ND5, COI and ND4 did not stain darkly with silver on this particular gel. (B) Solubilized mitochondria (15 μg) from ethanol-fed and control rats, stained with silver.

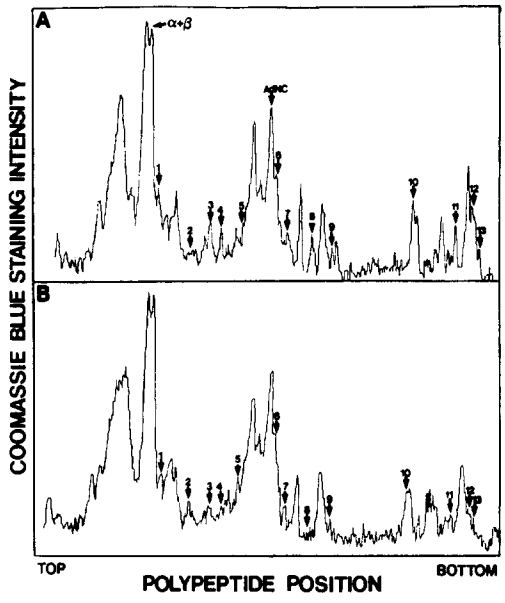

Fig. 7. Densitometric scans of gel patterns of SMP from an ethanol-fed rat and its pair-fed control. (A) SMP from an ethanol-fed rat. (B) SMP from the pair-fed control. Quantitation of the Coomassie blue staining intensity of the mitochondrial gene products is described in Materials and Methods. With each set of scans those portions where peaks of interest do not appear well resolved were displayed on an expanded scale to allow a better visualization of the individual peaks. This allowed better control in establishing integration parameters (selection of peak maxima and minima) which were then utilized for both the ethanol and control gel scans.

Solubilized mitochondria from ethanol-fed and control rats were also analyzed by SDS-PAGE with identical protein loads and Coomassie blue staining to assess the abundance of the mitochondrial gene products in the unfractionated organelle. The differences were less apparent than those observed with SMP due to the increased number of polypeptides in the intact mitochondrion. However, as can be seen in Fig. 6B, the concentration of mitochondrial gene product ND3 is reproducibly diminished in mitochondrial preparations

from ethanol-fed rats. Densitometric analyses of this polypeptide in mitochondrial preparations separated by SDS-PAGE gave values of 630 ± 60 and 963 ± 72 (relative units) for ethanol and control preparations, respectively ($n = 6$ pairs). These values reflect a 35 ± 3% decrease in the abundance of this mitochondrial gene product due to chronic ethanol consumption ($P = 0.004$). In contrast, densitometric analyses of the ($\alpha + \beta$) subunits of the $F_0 \cdot F_1$-ATPase in the same preparations of ethanol and control mitochondria gave values of 1577 ± 76 and 1532 ± 77, respectively ($n = 6$ pairs). Thus, the same preparations of mitochondria from ethanol-fed and control rats display a decrease in the concentration of mitochondrial gene product ND3, but no decrease in the concentrations of cytoplasmically synthesized subunits ($\alpha + \beta$) of the $F_0 \cdot F_1$-ATPase. Furthermore, densitometric analyses of the band for the adenine nucleotide carrier revealed that the absolute intensities were not significantly different in patterns from ethanol-fed and control SMP (data not shown). This observation confirms that the concentration of this nuclear gene product was not altered by chronic ethanol consumption. These observations appear to preclude the possibility that the lowered amounts of mitochondrial gene products in SMP are simply due to an ethanol-related problem of insertion of the polypeptides into the inner membrane.

The results of the current studies are summarized in Table I. The data in this table show the relationships between the observed depressions in radiolabeling and steady-state polypeptide abundance of the mitochondrial gene products with the reported depressions in holoenzyme activities [1–8]. With the majority of the mitochondrial gene products there is good correlation between loss in activity and the percentage depression in concentration and incorporation of [^{35}S]methionine. With ND5, ND4, ND2, COII and COIII the correlation between incorporation of radiolabel and abundance of stained polypeptide was not as strong. However, these moderate deviations may reflect less precision inherent in integrating the areas under peaks which were adjacent to those from prominent polypeptides. Nevertheless, the data in Table I for the ethanol-induced decreases in relative polypeptide concentration and relative rates of polypeptide synthesis ([^{35}S]methionine incorporation) correlate reasonably well with the reported depressions in holoenzyme activities or spectrophotometrically determined cytochrome content [1–8].

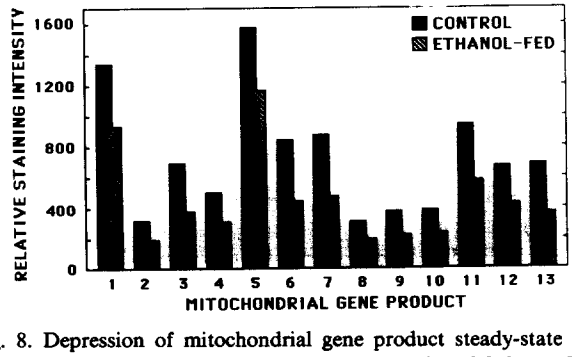

Fig. 8. Depression of mitochondrial gene product steady-state concentrations in submitochondrial particles from ethanol-fed rats. Each bar represents the average densitometrically determined Coomassie blue staining intensity from at least twelve animals for each of the mitochondrial gene products. The maximal standard error for these measurements was 9% of the mean value. Differences between ethanol and control values were determined to be significantly different for each of the mitochondrial gene products by the paired t-test ($P \leq 0.046$) and ANOVA statistical analyses ($P \leq 0.05$).

TABLE I

Relationships among the ethanol-induced depressions in polypeptide synthesis, polypeptide mass and enzymatic activity

Polypeptide number	Polypeptide	%Depression		
		radiolabel [a]	mass [a]	activity
1	ND5	50 ± 3	29 ± 3	40–50 [b]
2	COI	32 ± 3	38 ± 2	50 [c]
3	ND4	51 ± 3	37 ± 3	40–50
4	Cyt b	34 ± 3	38 ± 3	42 [d]
5	ND2	45 ± 2	26 ± 2	40–50
6	ND1	44 ± 3	44 ± 3	40–50
7	COII	33 ± 2	44 ± 3	50
8	COIII	48 ± 2	37 ± 3	50
9	ATPase 6	41 ± 3	40 ± 4	35 [e]
10	ND6	41 ± 3	39 ± 3	40–50
11	ND3	38 ± 3	38 ± 3	40–50
12	ATPase 8	41 ± 1	34 ± 3	35
13	ND4L	42 ± 2	45 ± 3	40–50

[a] Data obtained from the analyses of SMP reported in Figs. 5 and 8.
[b] Activity associated with polypeptides designated by ND was that of the NADH-ubiquinone reductase segment of the electron transport chain [1–4].
[c] Activity associated with polypeptides designated by CO was that of cytochrome c oxidase [1,4–6].
[d] Reported decrease in cytochrome b heme content [6,8].
[e] Activity associated with the ATPase subunits was that of the oligomycin-sensitive ATPase [7].

Discussion

The biogenesis of the mitochondrial oxidative phosphorylation system depends on contributions from the protein synthesizing systems of both the cytoplasm and mitochondrion. The mammalian mitochondrial genome encodes only thirteen open reading frames in addition to two rRNAs and a complete set of tRNAs [33]. It has now been well established that these open reading frames encode two polypeptides of the $F_0 \cdot F_1$-ATPase [34], cytochrome oxidase subunits I–III [25,27], apocytochrome b [25] and seven of the polypeptides of the NADH dehydrogenase complex [35–38]. The polypeptide products of mitochondrial protein synthesis are absolutely required for the proper assembly and function of the oxidative phosphorylation system [39]. Therefore, an ethanol-induced effect on mitochondrial biogenesis at

the level of mitochondrial protein synthesis might, over time, adversely affect the functioning of the mitochondrial oxidative phosphorylation system.

The observations in the present study indicate that chronic ethanol consumption depresses the incorporation of radiolabel into all thirteen mitochondrial gene products in vitro. These observations extend previous studies [10–12] which demonstrated that chronic ethanol consumption affects the capacity of isolated mitochondria to incorporate radiolabeled amino acids into total mitochondrial protein. The depression of all mitochondrial gene products appears to be coordinated since the mitochondrial gene products are synthesized in normal ratios. In addition, the results of the present study show that ethanol-fed rats contain lowered steady-state concentrations of the mitochondrial gene products. These observations allow the possibility of ethanol-related alterations at any step in mitochondrial protein synthesis preceding translation of an individual mature mRNA. The possibilities include ethanol-related alterations in DNA replication, transcription or construction of the functioning mitochondrial ribosome. Recent studies in our laboratory (Coleman and Cunningham, unpublished) have revealed that the concentration of competent ribosomes is depressed approx. 40% in mitochondria from ethanol-fed rats. It is likely that this decreased level of ribosomes affects both the rate of synthesis and steady-state concentrations of the mitochondrial gene products in ethanol mitochondria.

The results of these studies provide an explanation for the reductions in oxidative phosphorylation observed in mitochondria from ethanol-fed rats [1–8]. The current studies included in this paper suggest strongly that these alterations in mitochondrial function are the direct result of ethanol-induced effects on mitochondrial biogenesis, specifically mitochondrial protein synthesis. Since the mitochondrial gene products are necessary for the normal biogenesis of the mitochondrial oxidative phosphorylation system, a reduction in the number of mitochondrially synthesized proteins in ethanol mitochondria would necessarily result in a reduction of the number of the complexes of the oxidative phosphorylation system which could be assembled completely. Therefore, the effect of chronic ethanol consumption would be to reduce the number of competent complexes in the mitochondrial membrane as normal turnover of the system occurred, resulting in a reduction in oxidative phosphorylation activity. It was previously demonstrated that the ethanol-related decrease in the rate of mitochondrial ATP synthesis was due to depressions in the activities of the NADH-ubiquinone, cytochrome oxidase and ATP synthase complexes [4], all of which contain mitochondrial gene products.

Previous observations have suggested that alterations in the lipid content of mitochondrial membranes might account for the functional alterations of the oxidative phosphorylation system. It is well established that chronic ethanol consumption results in changes in the fatty acid composition of mitochondrial phospholipids [40]. This provides the possibility that the depression in the functioning of the oxidative phosphorylation system is attributable to these alterations in membrane lipids. Arai et al. [41] have provided evidence suggesting that changes in phospholipids may play a minor role in lowering the activity of cytochrome oxidase. However, other studies argue strongly that ethanol-elicited changes in phospholipids are not responsible for the observed decreases in the rate of ATP synthesis with either succinate [13] or glutamate [42] as oxidizable substrate. Moreover, the observations made in the present investigation indicate strongly that the depression in the functioning of the hepatic oxidative phosphorylation system can be accounted for by the alterations in the content of mitochondrial gene products in the mitochondrial inner membrane. The basis for this conclusion is the strong correlation between the altered protein content of ethanol mitochondria and the ethanol-elicited depressions in the corresponding segments of the oxidative phosphorylation system (Table I).

The ethanol-elicited alterations in the levels of mitochondrial gene products, which appear to be related to the decreased function of the oxidative phosphorylation system, may influence the overall energy state of the liver. It has been previously reported that chronic ethanol consumption decreases the hepatic energy state, as indicated by shifts in the adenine nucleotide distribution and increases in inorganic phosphate concentrations [43–45]. These alterations in hepatic energy state could be the result of increased utilization of ATP or a decrease in its synthesis. The results of the present study provide a partial explanation for the earlier observations which demonstrated a decrease in synthesis of ATP via the oxidative phosphorylation system. They also reaffirm that decreased synthesis of ATP has to be considered as a factor contributing to alterations in hepatic energy metabolism observed in animals subjected to chronic ethanol consumption. Alterations in the energy state of the hepatic tissue impinge upon at least two of the mechanisms suggested to be involved in the development of alcohol-induced liver disease: pericentral hypoxia [46] and decreased polymerization of tubulin [47]. Thus, alterations in mitochondrial function induced by chronic ethanol consumption have to be considered as factors important in the development of alcoholic liver disease.

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References

1 Bernstein, J.D. and Penniall, R. (1978) Biochem. Pharm. 27, 2337–2342.
2 Thayer, W., Ohnishi, T. and Rubin, E. (1980) Biochim. Biophys. Acta 591, 22–36.
3 Bottenus, R., Spach, P.I., Filus, S. and Cunningham, C.C. (1982) Biochem. Biophys. Res. Commun. 105, 1368–1373.
4 Spach, P.I. and Cunningham, C.C. (1987) Biochim. Biophys. Acta 894, 460–467.
5 Thayer, W. and Rubin, E. (1979) J. Biol. Chem. 254, 7717–7723.
6 Thayer, W. and Rubin, E. (1981) J. Biol. Chem. 256, 6090–6097.
7 Montgomery, R.I., Coleman, W.B., Eble, K.S. and Cunningham, C.C. (1987) J. Biol. Chem. 262, 13285–13289.
8 Thayer, W. and Rubin, E. (1982) Biochim. Biophys. Acta 721, 328–335.
9 Spach, P.I., Bottenus, R.E. and Cunningham, C.C. (1982) Biochem. J. 202, 445–452.
10 Hofmann, I. and Hosein, E.A. (1978) Biochem. Pharm. 27, 457–463.
11 Bernstein, J.D. and Penniall, R. (1978) Alcohol. Clin. Exp. Res. 2, 301–310.
12 Burke, J.P. and Rubin, E. (1979) Lab. Invest. 41, 393–400.
13 Spach, P.I., Parce, J.W. and Cunningham, C.C. (1979) Biochem. J. 178, 23–33.
14 Brown, R., Montgomery, R., Spach, P.I. and Cunningham, C.C. (1985) Biochem. J. 225, 597–608.
15 Lieber, C.S. and DeCarli, L.M. (1982) Alcohol. Clin. Exp. Res. 6, 523–531.
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.I. (1951) J. Biol. Chem. 193, 265–275.
17 Mills, N.C., Ray, D.B., Littlejohn, R.A., Horst, I.A. and Kowal, J. (1983) Anal. Biochem. 138, 164–180.
18 Faires, R.A. and Boswell, G.G.J. (1981) in Radioisotope Laboratory Techniques, 4th Edn., pp. 257–259, Butterworths, Boston.
19 Laemmli, U.K. (1970) Nature 227, 680–685.
20 Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.
21 Bhat, N.K., Niranjan, B.G. and Avadhani, N.G. (1982) Biochemistry 21, 2452–2460.
22 Mariottini, P., Chomyn, A., Doolittle, R.F. and Attardi, G. (1986) J. Biol. Chem. 261, 3355–3362.
23 Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature 290, 457–465.
24 Bibb, M.J., Van Etten, R.A., Wright, C.T., Walberg, M.W. and Clayton, D.A. (1981) Cell 26, 167–180.
25 Grosskopf, R. and Feldmann, H. (1981) Curr. Genet. 4, 151–158.
26 Anderson, S., De Bruijn, M.H., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. (1982) J. Mol. Biol. 156, 683–717.
27 Pepe, G., Holtrop, M., Gadaleta, G., Kroon, A.M., Cantatore, P., Gallerani, R., De Benedetto, C., Quagliariello, C., Sbisa, E. and Saccone, C. (1983) Biochem. Int. 6, 553–563.
28 Luzikov, V.N. (1985) in Mitochondrial Biogenesis and Breakdown (Roodyn, D.B., ed.), p. 155, Consultants Bureau, New York.
29 Thayer, W. and Rubin, E. (1986) Biochim. Biophys. Acta 849, 366–373.
30 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
31 Cederbaum, A.I., Lieber, C.S. and Rubin, E. (1974) Arch. Biochem. Biophys. 165, 560–569.
32 Mutvei, A. and Nelson, B. (1982) Biochem. Biophys. Res. Commun. 105, 916–921.
33 Tzagoloff, A. and Myers, A. (1986) Annu. Rev. Biochem. 55, 249–285.
34 Fearnley, I.M. and Walker, J.E. (1986) EMBO J. 5, 2003–2008.
35 Chomyn, A., Mariottini, P., Cleeter, M., Ragan, C., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. and Attardi, G. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5535–5539.
36 Chomyn, A., Mariottini, P., Cleeter, M., Ragan, C., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. and Attardi, G. (1985) Nature 314, 592–597.
37 Mariottini, P., Chomyn, A., Riley, M., Cottrell, B., Doolittle, R.F. and Attardi, G. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 1563–1567.
38 Chomyn, A., Cleeter, M., Ragan, C., Riley, M., Doolittle, R. and Attardi, G. (1986) Science 234, 614–618.
39 Attardi, G. and Schatz, G. (1988) Annu. Rev. Cell. Biol. 4, 289–333.
40 Cunningham, C.C. and Spach, P.I. (1987) Ann. N.Y. Acad. Sci. 492, 181–192.
41 Arai, M., Gordon, E.R. and Lieber, C.S. (1984) Biochim. Biophys. Acta 797, 320–327.
42 Gordon, E.R., Rochman, J., Arai, M. and Lieber, C.S. (1982) Science 216, 1319–1321.
43 Bernstein, J., Videla, L. and Israel, Y. (1973) Biochem. J. 134, 515–521.
44 Gordon, E. (1973) J. Biol. Chem. 248, 8271–8280.
45 Gillam, E. and Ward, L. (1986) Int. J. Biochem. 18, 1031–1038.
46 Israel, Y. and Orrego, H. (1987) Ann. N.Y. Acad. Sci. 492, 303–332.
47 Matsuda, Y., Baraona, E., Salaspuro, M. and Lieber, C.S. (1979) Lab. Invest. 41, 455–463.