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EFFECTS OF CHRONIC ETHANOL CONSUMPTION ON THE RATE OF RAT LIVER MITOCHONDRIAL PROTEIN TURNOVER AND SYNTHESIS

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Abstract—Incorporation *in vitro* of labeled leucine into isolated rat liver mitochondria was decreased in animals chronically fed ethanol. To avoid artifactual leucine incorporation, no trichloroacetic acid (TCA) precipitation was performed. Further, it was found that when these mitochondrial proteins were separated on sodium dodecyl sulfate polyacrylamide gels, no specific reduction of leucine incorporation into any individual protein bands could be detected. This probably reflected a general decrease in amino acid incorporation occurring in these isolated mitochondria. Incorporation *in vivo* of labeled arginine detected a decrease in the viability of rat liver mitochondria from chronic ethanol-fed rats. This incorporation revealed a 2-day decrease in the half-life of liver mitochondria from chronic ethanol-fed rats. It was also found that arginine incorporation by the cytoplasmic protein-synthesizing system is increased *in vivo* after chronic ethanol feeding. The results from these experiments suggest that if this reaction were to persist through continuous consumption of ethanol, there would in time be diminished amounts of properly assembled proteins which are necessary for membrane structure and function. Whether or not such induced deficiencies in the mitochondria over a long period of time are precursors for alcoholic hepatitis and/or cirrhosis remains to be explained.

Mitochondria isolated from the livers of rats chronically fed ethanol show distinct morphologic changes [1–3] which have been attributed to altered protein synthesis in both the mitochondrial and the cytoplasmic ribosomes [4, 5]. It has been suggested that with continuous exposure to large doses of ethanol, mitochondrial membrane biogenesis may be adversely affected [4], leading to or causing the morphologic changes observed in such mitochondria. Mitochondrial protein synthesis, which is distinct from cytoplasmic protein synthesis, is believed to be responsible for the synthesis of certain components of mitochondrial enzymes [6–10]. A decrease in the synthesis of such proteins may possibly account for the changes observed with electron microscopy in liver mitochondria from rats chronically fed ethanol [1, 4]. The assembly of the lipids into these membranes appears to occur at the same rate and simultaneously with the proteins [11]; thus the incorporation of leucine into mitochondrial proteins may be considered an accurate measure of mitochondrial membrane biogenesis.

When Schimke *et al.* [12] studied the turnover rate of rat liver mitochondrial proteins with labeled arginine, they correlated this with the actual turnover rate of these organelles. They found it to be approximately 6.8 days. Because of the effects seen by other investigators in liver mitochondria after chronic ethanol administration, it was of interest to determine how an overall decrease in mitochondrial protein synthesis might affect the protein turnover rate of mitochondria harvested from Metrecal-fed controls and chronic ethanol-fed rat livers. Also, we determined whether the decrease in mitochondrial leucine incorporation represented selective inhibition of leucine incorporation into certain individual proteins or a decrease in total incorporation of labeled leucine in mitochondrial proteins.

METHODS

Chronic ethanol feeding and preparation of liver mitochondria. Male Sprague–Dawley rats weighing approximately 150 g were fed a diet of Metrecal (Mead-Johnson) and those animals selected as the experimentals were fed ethanol which was added to the diet [13]. Chronic ethanol feeding was achieved with a Metrecal–ethanol liquid diet in which ethanol provided 37 per cent of the calories, protein 16 per cent, fat 5 per cent and the remaining calories from carbohydrate. This diet facilitated incorporation of all required nutrients plus ethanol into the liquid. With control animals, an isocaloric amount of sucrose replaced the ethanol. The ethanol-containing diet was introduced gradually to the rats in week 1, so that at the end of that week, a level of 37 per cent of the total calories consumed was obtained from ethanol. This diet was maintained for a minimum of 4 weeks. On the day of the assay, both Metrecal-fed (control) and Metrecal–ethanol pair-fed animals were killed, the livers removed and quickly placed in beakers containing 8 vols: 250 mM sucrose; 5 mM Tris–HCl, pH 7.4; and 1 mM EDTA (ST-EDTA). Each liver was homogenized briefly (15 sec) and centrifuged at 3000 *g* for 1 min. The supernatant 1 was collected and kept cold while the pellet was resuspended in eight times its volume of ST–EDTA and recentrifuged at 3000 *g* for 1 min. The pellet was discarded and supernatant 2 formed combined with the supernatant 1 was centrifuged at 27,000 *g* for 3 min. The supernatant 3 was discarded, the pellet resuspended in 20 vols ST (250 mM sucrose; 5 mM

Tris–HCl, pH 7.4) and centrifuged at 3000 *g* for 1 min. The pellet was discarded and supernatant 4 recentrifuged at 27,000 *g* for 3 min. The supernatant 5 was discarded. The pellet contains the mitochondria and a pink layer of microsomes which was removed by decanting.

The final mitochondrial pellet was contaminated by less than 3% microsomal protein when assayed for glucose 6-phosphatase activity as described by Beattie [14].

Mitochondrial integrity. It has been established that intact liver mitochondria are nearly totally impermeable to exogenous NADH [15]. The property of NADH therefore makes it very suitable to test mitochondrial integrity. The assay system devised by Lehninger [16] was used for this work. NADH, which absorbs maximally at 340 nm, was used as substrate for D-β-hydroxybutyric, malic and glutamic dehydrogenases [17, 18]. The decrease in absorbance at 340 nm is directly proportional to the rate of oxidation of NADH.

State 3 and 4 respiration. Oxygen was measured poloragraphically on a Fisher series 5000 recorder with intact mitochondrial samples of 1 ml using standard Clark electrodes, assuming a constant pressure of 1 atm. The activity of the succinoxidase system (state 4) was determined in an incubation mixture containing: 0.25 M sucrose, 0.01 M Tris, 0.01 m K_2HPO_4, 50 mM $MgCl_2$, 50 mM succinate and 0.5 mg/ml of bovine serum albumin, adjusted to pH 7.4.

State 3 oxygen uptake was determined in separate experiments with the addition of 0.25 ml ADP (1.63 mg/ml) to the state 4 system.

The ratios of oxygen uptake were calculated from the activity slopes obtained in the initial 60 sec.

Bacterial contamination. All the steps involving mitochondria and mitochondrial amino acid incorporation *in vitro* were performed under strictly sterile conditions. In all cases, glassware and solutions, where possible, were autoclaved or filtered through 0.20-μm millipore filters into sterile containers prior to use (Nalge Sybron Corp., Rochester, NY). Invariably after each incubation *in vitro*, a sample of the incubation medium was plated on Triptone Soy Agar (TSA) sheep blood–agar plates and incubated for 72 hr at 37° to estimate the extent of bacterial contamination.

Arginase assay. Assays for arginase activity were performed on rat liver homogenates as described by Brown and Cohen [19].

Leucine incorporation studies in vitro. The mitochondrial pellet was resuspended in ST and diluted to a final protein concentration of 2.5 mg/ml. One-ml aliquots of this tissue suspension were then mixed with 1 ml of incubation medium to give a solution with the following composition: 50 mM Bicine (pH 7.6), 10 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 2 mM ATP, 1 mM EDTA, 10 μg/ml of pyruvate kinase, 65 mM KCl, 22.5 mg/ml of amino acid mixture minus leucine and 100 μg/ml of cycloheximide [20].

The suspension was then preincubated at 30° for 3 min in a shaking water bath to permit temperature equilibration. The reaction was initiated on addition of 20 μCi L-leucine [^{14}C(U)] or 40 μCi L-leucine [4,5-^{3}H(N)] (New England Nuclear, Lachine, Quebec). The specific activity of the [^{14}C]leucine and the [^{3}H]leucine was 303 mCi/m-mole and 42 Ci/m-mole respectively. One hr later the reaction was terminated by addition of an equal volume of ice-cold unlabeled leucine (final concn 10 mM) in ST. The flasks were immediately removed from the water bath and placed on crushed ice. The mitochondria were then reisolated by centrifugation at 27,000 *g* for 3 min and washed twice with the cold unlabeled leucine-ST.

Gel electrophoresis. The washed mitochondrial pellets obtained as described above were resuspended in ST and diluted to a protein concentration of 2 mg/ml. This suspension was then added to an SDS (sodium dodecyl sulfate) sample medium solution containing a final concentration of: 62.5 mM Tris–HCl, pH 6.8; 2% SDS, 10% glycerol; and 5% mercaptoethanol.

To ensure monomerization and prevent proteolysis, 1 mM phenylmethyl sulfoxyl fluoride was added and the mixture was heated at 70° for 20 min with vigorous mixing [21]. The proteins were separated on 10-cm gels made up of 3% stacking and 10% running gels [22]. Electrophoresis was carried out at room temperature for 3–4 hr. The gels were stained overnight with 0.025% Coomassie blue in isopropyl alcohol–acetic acid–water (25:10:65). They were destained on shaking with 0.0025% Coomassie blue in isopropanol–acetic acid–water (10:10:80) for 6–9 hr and then overnight in 0.0025% Coomassie blue in 10% acetic acid. Final destaining was done in 10% acetic acid. The stained gels were scanned at 540 nm in a Gilford spectrophotometer.

Gel slicing and counting. The gels were cut into 2-mm sections with a Gilson Gel Slicer and washed with distilled H_2O directly into counting vials. The samples were dried in an oven at 45° until the gels were only slightly moist and 0.2 ml hydrogen peroxide was added. The gels were then kept at 50° overnight and cooled prior to addition of 10 ml of Hydromix scintillation counting mixture (Canatech. Inc., Montreal, Quebec). Radioactivity was determined in an Intertechnique scintillation counter for both the ^{3}H- and ^{14}C-labeled leucine, and corrections for quenching, spill and efficiency were applied by a Multimat computer to present the data in dis./min and ratios of ^{3}H/^{14}C for each sample.

Criteria for defining a peptide band whose synthesis is induced or repressed. The % ^{3}H to % ^{14}C ratio was calculated for the mean ^{3}H/^{14}C ratio. This calculation was performed to permit comparison between experiments even when the mean ratios varied. The Y axis in Fig. 1(a) is expressed in % ^{3}H/% ^{14}C. An altered ratio in a particular band can reflect either a random deviation from the mean ratio (1.0) or a protein band which is induced or repressed. We have defined the cutoff point as ±2 standard deviations (S.D.) (±0.41). Any polypeptide with a reproducible isotope ratio greater than 2 S.D. was considered as either induced or repressed. This criterion was used so that it would be highly improbable that such a large reproducible deviation could be due to a random variation from the mean isotope ratio.

Protein analysis. Protein concentrations were determined by the method of Lowry *et al.* [23].

Studies in vivo. Rats weighing 250 g maintained on a Metrecal or Metrecal–ethanol diet described above

received on day 0 a single 1-ml intraperitoneal injection of L-arginine guanido[^{14}C] (10 μCi), with a specific activity of 23 mCi/m-mole. They were then maintained on their respective diets until sacrificed. After sacrifice, the liver was removed and the mitochondria were isolated as described above. The mitochondrial pellets were suspended in distilled H_2O. One-ml aliquots were dissolved in 10 ml Hydromix and counted in an Intertechnique scintillation counter.

RESULTS

Mitochondrial preparations were studied to determine whether they manifested any structural changes as assessed with the classical assays which characterize intactness and damage. Mitochondria from both control and chronic ethanol-fed rats were incubated with NADH under state 4 conditions (no ADP), and disappearance of NADH was followed spectroscopically at 340 nm. Control mitochondria had a rate of oxidation of 3.25 $\pm$ 0.48 nmoles NADH/mg of protein/min while chronic alcoholic mitochondria oxidized 3.00 $\pm$ 0.53 nmoles NADH/mg of protein/min. Sonicated preparations of both control and alcoholic mitochondria had a value of 53 $\pm$ 13 and 48 $\pm$ 11 nmoles NADH/mg of protein/min respectively. Because values for ethanol-fed rat liver mitochondria agree closely with those for control intact mitochondria, and the difference between the two preparations is not statistically significant, we assumed that the mitochondrial membrane permeability to NADH as assessed by NADH oxidation did not change whith chronic ethanol feeding.

Similar preparations of these mitochondria were also assayed under conditions which measure state 3 and 4 respiration. The values of the respiratory control ratio (RCR) with succinate were 3.61 $\pm$ 0.35 for control mitochondria and 3.41 $\pm$ 0.49 for chronic ethanol-fed rat liver mitochondria. A 30 per cent decrease in succinate respiration by mitochondria from chronically ethanol-fed rat livers did not affect the RCR of these mitochondria. The RCR from both groups of rat liver mitochondria was the same.

Leucine incorporation in vitro *by isolated mitochondria.* There are two possible explanations for the decrease in leucine incorporation *in vitro* reported by Rubin *et al.* [4] in liver mitochondria from chronic ethanol-fed rats, provided the artifactual effect of trichloroacetic acid (TCA) described by Hochberg *et al.* [24] was eliminated. The first possibility is that a general overall decrease in mitochondrial leucine incorporation might be observed in the chronic ethanol-treated animals.

Second, a decreased incorporation into one or more individual proteins (i.e. induction or repression of individual polypeptides) may be recorded as a fall in total leucine incorporation. It was therefore our first objective to establish whether this artifact of TCA protein precipitation affected the results obtained in these mitochondria, and our second to establish whether a general or a specific decrease in mitochondrial leucine incorporation occurred after chronic ethanol feeding. In the experiments involving the incorporation of labeled leucine, the reactions were terminated by addition of an ice-cold unlabeled leucine–sucrose–Tris (pH 7.4) solution, followed by washing the mitochondria three times in this solution.

Determination of the extent of bacterial contamination on the various mitochondrial preparations after incubation of agar plates for 72 hr at 37° showed that it was possible to detect from 100 to 500 colonies of bacteria/incubation. This number of bacteria is considered insignificant in affecting the incorporation of labeled leucine into the protein synthesized by these mitochondria [21].

When chloramphenicol is added to the incubation medium, it can be seen from the results shown in Table 1 that a fall of 75–86 per cent in the incorporation of both ^{3}H- and ^{14}C-labeled leucine occurred. Unlike the possible artifactual results described by Hochberg *et al.* [24] with TCA protein precipitation, the incorporation of labeled leucine in these mitochondria is very sensitive to inhibition by chloramphenicol. Also, it may be observed in Table 1 that a decrease of 33–37 per cent in the total incorporation of both [^{3}H]- and [^{14}C]leucine occurred with mito-

Table 1. Labeled leucine incorporation into isolated rat liver mitochondria

Treatment *in vivo*	Additions *in vitro*	Rate of incorporation (dis./min/mg protein/min)
Control*	[^{14}C]leucine	434 $\pm$ 17†
	[^{3}H]leucine	1297 $\pm$ 90‡
	[^{14}C]leucine + chloramphenicol	62 $\pm$ 16
	[^{3}H]leucine + chloramphenicol	328 $\pm$ 30
Chronic ethanol*	[^{14}C]leucine	275 $\pm$ 22†
	[^{3}H]leucine	871 $\pm$ 88‡
	[^{14}C]leucine + chloramphenicol	39 $\pm$ 10
	[^{3}H]leucine + chloramphenicol	202 $\pm$ 32

* Mean of six animal mitochondrial preparations.

† [^{14}C]leucine incorporation between controls and experimentals significant at $P < 0.01$ level.

‡ [^{3}H]leucine incorporation between controls and experimentals significant at $P < 0.01$ level.

chondria isolated from chronic ethanol-fed rat livers. This decreased leucine incorporation is in agreement with Rubin *et al.* [4], who found a similar decrease in labeled leucine incorporation. It should be noted that, in the present experiment, no TCA was used to terminate the reaction, thus eliminating the possibility of artifactual incorporation of labeled leucine into the protein by this reagent.

Liver mitochondrial pellets from both control and chronic ethanol-fed rats were incubated with [^{3}H]- and/or [^{14}C]leucine. The control [^{3}H]leucine-labeled pellet was then mixed with the [^{14}C]leucine-labeled chronic ethanol-fed pellet and *vice versa*. The experiments using the reverse labeled isotopes were performed to avoid the kind of artifactual results described by Ramirez *et al.* [25]. These pellets were then dissolved in the SDS sample medium and applied on the SDS polyacrylamide gels. The resultant gels were scanned at 540 nm after staining and destaining. This revealed that the entire sample penetrated the gel and that it was separated into approximately 25 distinct bands. The gels were then sliced, dissolved and counted for both [^{3}H]- and [^{14}C]leucine incorporation. A gel scan, Fig. 1(b), of leucine incorporation was constructed and from this is can be seen that 12 of the polypeptide bands incorporated the labeled leucine. This number of labeled bands is in agreement with Schatz and Mason [26], who claim that rat liver mitochondria synthesize no more than a dozen polypeptides.

By densitometry this scan revealed that no peaks were absent in either the [^{14}C]- or [^{3}H]-labeled alcoholic mitochondria. Both ethanol-fed and control incorporation peaks, whether labeled with [^{3}H] or [^{14}C], matched exactly. When the weighted ratio of %[^{3}H]/%[^{14}C] is calculated and plotted in Fig. 1(a), it can be seen that only peaks 9 and 10 fall outside the range of one standard deviation, but they both fall within two standard deviations. Variations in the ratios of these leucine incorporation peaks do not represent significant deviations from the mean. No peak in the proteins synthesized by the ethanol-fed mitochondrial preparation shows any specific increase or decrease of leucine incorporation. It may be concluded that the decrease in total leucine incorporation observed in the chronic ethanol-fed mitochondria was probably due to an overall decrease in the amino acids incorporated into these proteins.

Attempts to resolve the large peak at the front of the gel failed to show any further resolution even when 20% polyacrylamide gels were prepared [27].

Turnover rate of mitochondrial proteins in vivo. A total of 24 rats was maintained on a Metrecal diet and 24 pair-fed partners on the chronic ethanol Metrecal diet. Four rats from each group were sacrificed at intervals of 1, 2, 3, 4, 5 and 8 days after labeled arginine injections. The mitochondria from these rat livers were isolated as described in Methods and an aliquot was counted for L-arginine guani-

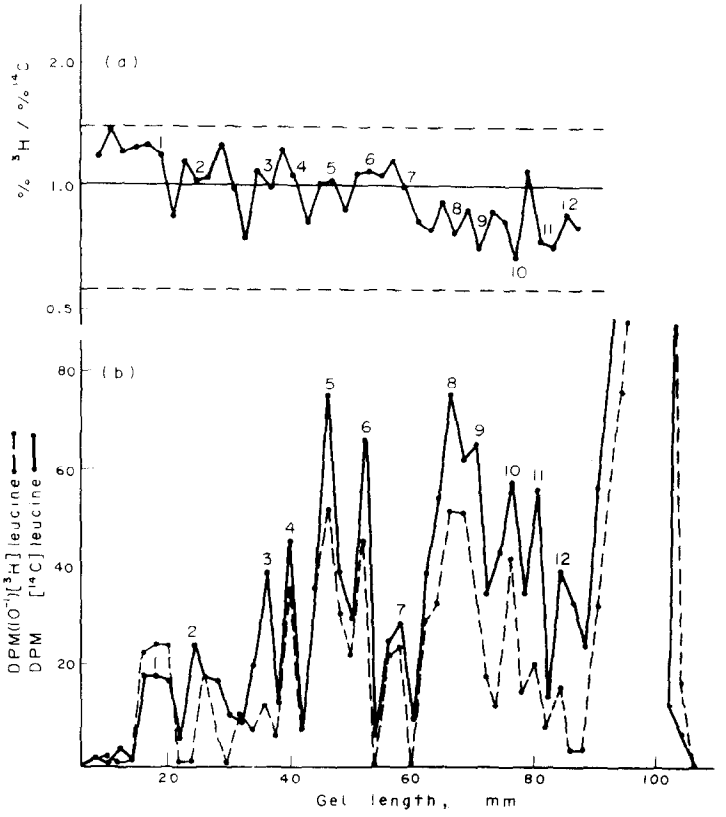

Fig. 1. (a) Weighted ratios of control [^{3}H]leucine and chronic ethanol-fed [^{14}C]leucine incorporation into proteins isolated from rat liver mitochondria and separated on SDS polyacrylamide gels. The dotted lines represent the limits of two standard deviations. (b) Control (-----) [^{3}H]leucine and chronic ethanol-fed (——) [^{14}C]leucine incorporation into proteins isolated from rat liver mitochondria, determined by liquid scintillation counting of solubilized 2-mm slices of SDS polyacrylamide gel.

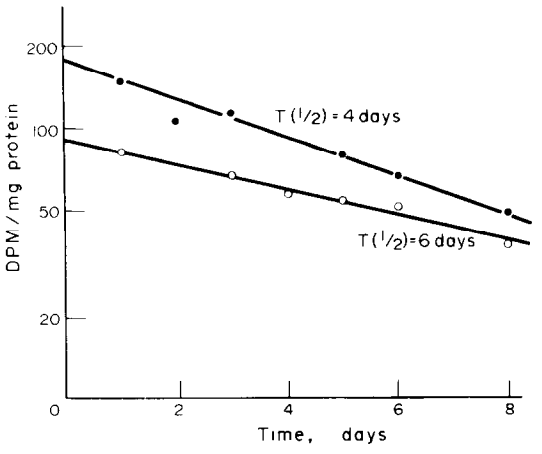

Fig. 2. Half-life of mitochondrial proteins determined by the plot of log L-arginine guanido [^{14}C] incorporation/mg of protein at various time intervals after labeled arginine injection into control (○——○) and chronic ethanol-fed rats (●——●).

do[^{14}C] incorporation. An aliquot was retained for protein estimation.

When total mitochondrial proteins in both mitochondrial preparations were compared, no significant difference could be detected. The control values for total proteins are 78 ± mg protein/g wet weight liver and the alcoholic values are 79 ± 6 mg protein/g wet weight liver.

The results of the arginine incorporation experiments are plotted in Fig. 2 and two observations can be readily made. First, the half-life of the chronic ethanol-fed rat liver mitochondrial proteins is approximately 2 days shorter than that of the Metrecal-fed controls. Second, when these graphs are extrapolated to day 0, the initial incorporation of arginine into chronic ethanol-fed mitochondrial proteins is approximately double that of the controls. A similar effect is shown in Table 2 with the whole homogenate, the microsomal fraction, and proteins in the supernatant prepared from these rat livers.

Since a decrease in the activity of the liver arginase could preferentially have made the substrate available to the protein-synthesizing system, its activity was assayed in both control and experimental livers. It was found that the arginase activity in the liver homogenates was not altered by the diets fed to these animals.

DISCUSSION

Incubation *in vitro* and subsequent gel electrophoresis of the amino acids incorporated by control and chronic ethanol-fed rat liver mitochondrial proteins revealed two main points. The incorporation of both [^{3}H]- and [^{14}C]leucine is significantly decreased in this system *in vitro* with liver mitochondria isolated from chronic ethanol-fed rats. This decreased incorporation measured without TCA precipitation agrees with Rubin *et al.* [4], who used TCA to precipitate the proteins in the presence of labeled leucine. Also, it was shown that unlike the results published by Hochberg *et al.* [24], these mitochondrial preparations were inhibited by chloramphenicol. Moreover, when polyacrylamide gels were run on these mitochondrial proteins after incubation with labeled leucine, it was observed that the decreased leucine incorporation in the liver mitochondria of the chronic ethanol-fed rats was probably due to a general overall decrease in leucine incorporation rather than specific decreased incorporation into one or more proteins. Since this defect in mitochondrial leucine incorporation is observed in chronic ethanol-fed rats, it is possible that the biogenesis of the mitochondrial membrane could eventually be affected in such animals [4]. This possibility was tested *in vivo* with the arginine incorporation experiment, where it was observed that mitochondria from control animals had a half-life of approximately 6 days and chronic ethanol treatment reduced this to approximately 4 days. These data obtained *in vivo* support the hypothesis that the defect(s) affecting amino acid incorporation found *in vitro* could, in the long run, affect the mitochondria or mitochondrial membrane biogenesis so as to reduce mitochondrial viability. No decrease in total mitochondrial proteins was detected in the alcoholic preparations. This is probably due to the fact that these animals were only exposed to the ethanol for very short periods of time. Extended periods of ethanol abuse could in time, through failure of this system, conceivably affect the total mitochondrial population and the viability of the whole liver cell, eventually leading to cirrhosis of the liver.

The increased incorporation of arginine into liver mitochondria *in vivo* may seem incompatible with the

Table 2. Turnover and incorporation of labeled arginine into various proteins of rat liver cell fractions

Liver fractions	$T_{\frac{1}{2}}$ (days)*	Extrapolated incorporation at day 0 (dis./min/mg protein)
Control homogenate	5.4 ± 0.3	100 ± 12
Alcoholic homogenate	4.3 ± 0.2	210 ± 18
Control mitochondria	6.3 ± 0.2	87 ± 10
Alcoholic mitochondria	4.2 ± 0.3	188 ± 16
Control microsomes	4.9 ± 0.3	52 ± 11
Alcoholic microsomes	2.3 ± 0.3	155 ± 15
Control supernatant	6.8 ± 0.2	130 ± 9
Alcoholic supernatant	3.5 ± 0.3	310 ± 22

* Mean value of six rats for each treatment

decreased leucine incorporation observed with isolated mitochondria *in vitro*. However, it should be recalled that with isolated mitochondria, only the mitochondrial protein-synthesizing system is operating, and it is responsible for the synthesis of less than 10 per cent the total protein in mitochondria [28]. Thus the activity of the cytoplasmic protein-synthesizing system, which is responsible for 90 per cent of the proteins incorporated into mitochondria, is being monitored in this experiment *in vivo*. It has been suggested that no appreciable problem with pool size occurs in the case of arginine because of high levels of hepatic arginase [29].

Any excess labeled arginine would be immediately converted to ornithine and urea, with the [^{14}C] label in the urea. Thus the extramitochondrial pool size of the leucine is regulated by the concentration of leucine in the incubation medium and that of arginine should be regulated by the arginase. Mitochondrial integrity seemed unaffected by chronic ethanol treatment when both NADH oxidation and RCR were measured and no change in total mitochondrial protein and arginase activity could be detected in both liver preparations. Without direct measurements it is difficult to state unequivocally that changes in pool size are not involved with the changes measured in amino acid incorporation experiments both *in vivo* and *in vitro*. However, from the indirect measurements mentioned above, it appears unlikely that changes in the pool sizes of these two amino acids would have affected the results observed in these experiments.

Kuriyama *et al.* [5] showed that the ribosomal system *in vitro* isolated from the liver of mice, after 2 weeks of chronic ethanol treatment, had a 30 per cent increase in leucine incorporation. The increase in the isolated protein-synthesizing system of Kuriyama *et al.* [5] may be analogous to the increased arginine incorporation observed in our studies *in vivo*. The increase in protein synthesis observed by Iseri *et al.* [3] and others [5, 30] leading to the increase in smooth endoplasmic reticulum and drug-metabolizing enzyme activity in the liver may also account for the enhancement of the microsomal ethanol-oxidizing system (MEOS) found by Lieber and De Carli [31] in chronic ethanol rat livers. Such induction of cytoplasmic protein synthesis [3, 5, 30, 31] may also be comparable with the synthesis of cytochrome P450 found in the liver after treatment of animals with certain drugs [32]. Thus, the mitochondrial and cytoplasmic protein-synthesizing systems in the liver responded differently to chronic ethanol treatment.

It seems unlikely that the decreased leucine incorporation in isolated mitochondria may be due to diminished availability of ATP, since this would also have affected the cytoplasmic system. It is therefore conceivable that the substrates needed for this incorporation had difficulty being transported through the mitochondrial membrane system. This could possibly be due to a decrease in membrane permeability similar to that observed by French and Tudoroff [33], who used phenazine methosulfate (PMS) to study this phenomenon in brain mitochondria of chronic ethanol-fed rats sampled at time zero.

It would be of considerable interest to determine the exact site or sites of action of chronic ethanol abuse on protein synthesis. When labeled amino acids are incubated *in vitro* with the mitochondria or administered *in vivo* by intraperitonial injection, many complex systems are involved in the synthesis of the proteins. Any one or a combination of these systems could be affected by chronic ethanol abuse. These include the transport of substrates such as nucleotides, amino acids, O_2, oxidizable substrates and ions into mitochondria or cells for experiments both *in vitro* and *in vivo* respectively. Ethanol abuse could also affect many mitochondrial enzymes such as DNA polymerase, RNA polymerase, activating enzymes, respiratory enzymes and electron transport chain enzymes, through either inhibition or alteration of their activity, thereby affecting the rate of amino acid incorporation into proteins.

It is also worthwhile noting that regardless of the site of action of ethanol, observations recorded in this paper regarding the incorporation of amino acids into protein will not be altered but merely qualified as to where this interference or enhancement is occurring.

The diminished intramitochondrial leucine incorporation may subsequently be reflected in the decreased half-life of the mitochondrial proteins and likely in the turnover of liver mitochondria from chronic ethanol-fed rats. Since the half-life of control mitochondria is approximately 6 days and that of chronic ethanol mitochondria is 4 days, after 4 weeks only 4 per cent of the original mitochondrial proteins remains in the control mitochondria due to turnover, while less than 1 per cent would be present in the mitochondria of chronic ethanol-fed rats. This seems to be a circular phenomenon in which the ethanol treatment affects the viability of these mitochondria. As the treatment progresses, it is conceivable that these effects could diminish the viability of these mitochondria. This could eventually lead to decreased numbers of intact and functional mitochondria in the chronic ethanol-treated animals. It would seem unlikely to find such a serious defect in the mitochondria used in the present study since the rats had received chronic ethanol feeding for less than 4 weeks. Irreversible liver damage in primates and humans requires years of chronic ethanol abuse [34]. A decrease in the total number of liver mitochondria in the present study was therefore not expected.

Mitochondrial protein synthesis has been shown to be responsible for the synthesis of several important membrane-bound enzymes including cytochrome oxidase [6, 7], oligomycin-sensitive ATPase [8, 9] and cytochrome *b* [10], all of which are indispensable for the biogenesis and proper metabolic functioning of the mitochondria [26]. The proteins synthesized in the cytoplasm require those synthesized inside the mitochondria for the proper assembly of the inner mitochondrial membrane enzymes mentioned above [35–40]. If the decrease in intramitochondrial leucine incorporation demonstrated *in vitro* with the ethanol-fed mitochondria can be extrapolated *in vivo*, it could affect the proper integration of cytoplasmic proteins into these organelles. This, along with the increased turnover of the alcoholic mitochondria, will affect the biogenesis and function of these membrane enzymes in spite of the apparent increase in arginine incorporation into the alcoholic rat liver mitochondria. In fact,

the activity of some inner membrane-bound enzymes is affected by chronic ethanol administration. Rubin *et al.* [4] and Cederbaum *et al.* [41] found that the activity of succinate dehydrogenase, cytochromes a–a_3, cytochrome b and certain other electron transport chain components in liver mitochondria [42] show a decrease in content or activity after chronic ethanol feeding to rats.

It might therefore be presumed that after 4 weeks of treatment with ethanol, most liver mitochondria would be, to some extent, deficient in certain proteins which might represent the early manifestations of chronic alcohol treatment. Whether or not an exaggeration of this sequence of events leads to necrosis of the liver cells and finally cirrhosis of the liver, remains to be established.

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