Effect of Chronic Ethanol Ingestion on Fatty Acid Oxidation by Hepatic Mitochondria*

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To study possible factors in the pathogenesis of the ethanol-induced fatty liver, we investigated the effect of chronic ethanol consumption on the metabolism of fatty acids by isolated hepatic mitochondria. Chronic ethanol consumption resulted in decreased fatty acid oxidation, as evidenced by a reduction in oxygen uptake and CO₂ production associated with the oxidation of fatty acids. The State 3 rate of oxygen uptake was depressed to a greater extent than the State 4 or the uncoupler-stimulated rate; the respiratory control ratio was also decreased. Therefore, one site of action of chronic ethanol feeding is on oxidative phosphorylation.

The reduction in fatty acid oxidation, in general, is not due to an effect on the activation or translocation of fatty acids into the mitochondria. There was no effect by ethanol feeding on the activity of palmitoyl coenzyme A synthetase, whereas carnitine palmitoyltransferase activity was increased. The use of an artificial system (formazan production) to study β oxidation in the absence of the electron transport chain is described. In the presence of fluorocitrate, which inhibits citric acid cycle activity, ketogenesis and formazan production were increased by chronic ethanol consumption. Thus β oxidation to the level of acetyl-CoA is not impaired by chronic ethanol consumption. Total oxidation of fatty acids to CO₂ is depressed by chronic ethanol intoxication because of effects on oxidative phosphorylation or the citric acid cycle (or both). Neither nutritional deficiency, cofactor depletion, nor the presence of ethanol in vitro explains these effects. Several of the effects of chronic ethanol consumption on fatty acid oxidation are mimicked by acetaldehyde and acetate, products of ethanol oxidation. Chronic ethanol consumption leads to persistent impairment of mitochondrial oxidation of fatty acids to CO₂. However, oxidation of fatty acids to acetyl-CoA is not decreased by chronic ethanol consumption.

Chronic ethanol consumption in animals (1–4) and man (2, 5, 6) leads to fatty liver, in the absence of nutritional deficiencies. Several mechanisms have been suggested for the ethanol-induced steatosis, including decreased fatty acid oxidation by mitochondria (7). The lipids deposited in the liver after chronic ethanol intoxication are principally of dietary origin (8), suggesting reduced hepatic oxidation of fatty acids. Ethanol metabolism has been reported to decrease ¹⁴CO₂ production from labeled palmitate and acetate in liver slices (7), which points to decreased β oxidation or reduced activity of the citric acid cycle (7) or both. The production of reducing equivalents by the oxidation of ethanol results in a lowering of the mitochondrial oxidation-reduction state, which causes reduction of oxalacetate to malate, thus decreasing activity of the citric acid cycle.

In contrast to acute ethanol intoxication, chronic ethanol ingestion is associated with striking ultrastructural changes in the mitochondria (9–11) and increased membrane fragility (12). In addition, selective mitochondrial functions such as oxygen consumption with a variety of substrates, oxidative phosphorylation, and energized Ca²⁺ uptake are impaired by chronic ethanol feeding (13). These findings suggested that chronically compromised integrity of the mitochondria, independent of the biochemical events associated with ethanol metabolism, might interfere with fatty acid oxidation. In this study we have continued our investigations into the pathogenesis of fatty liver by determining the effect of chronic ethanol ingestion on the metabolism of fatty acids by isolated hepatic mitochondria.

MATERIALS AND METHODS

Preparations—Male Sprague-Dawley rats, weighing about 150 g, were fed for 24 days a nutritionally adequate liquid diet (14), in which carbohydrate provided 47% of total calories, protein 18%, and fat 35%. Pair-fed littermates consumed the same diet except that ethanol isocalorically replaced carbohydrate, accounting for 36% of total calories. The hepatic triglyceride content increased about 6-fold after ethanol feeding (14). Total hepatic protein and mitochondrial protein per g of liver were not affected by ethanol feeding (15). Liver mitochondria were prepared in 0.25 M sucrose-0.01 M Tris-HCl, pH 7.4-0.001 M EDTA, as previously described (16). Radioactive
The upper layer of acetone, which contained the methylene blue, was discarded. The amount of formazan produced was calculated from the centrifugation, the reduced formazan was retained in the CCl₃ phase.

...oxidation, namely, fatty acyl-CoA dehydrogenase and P-hydroxy-...acids by a modification of the original method of Mii and...in triplicate, each experiment having its own control, to which no fatty...formazan, served as an intermediate acceptor in the artificial electron acceptor system. Among these are reducing enzymes in mitochondria and in cell-free homogenates, the enzymes in mitochondria and in cell-free homogenates, the content of mitochondrial protein (milligrams per g wet weight) or the percentage of yield of mitochondria was the same for mitochondria from ethanol-fed rats as in controls. Mitochondria from ethanol-fed rats contained cytochromes b, c₁, a, and a₃ (although the content of cytochromes was lower (11)) as well as classical mitochondrial marker enzymes such as succinic dehydrogenase, α-glycero-phosphate oxidase, and cytochrome oxidase (although the specific activity of these enzymes was lower (15)). Indeed, by assaying the activity of these enzymes in mitochondria and in cell-free homogenates, the content of mitochondrial protein (milligrams per g wet weight) or the percentage of yield of mitochondria was the same for mitochondria from ethanol-fed rats as in controls. (15). It, therefore, appears that chronic ethanol feeding did not grossly disrupt the liver so that proteins isolated as mitochondrial proteins do not actually represent true mitochondrial proteins, i.e. the enzyme activities assayed in vitro appear to represent those found in situ. The ethanol-induced injury to mitochondria previously described (11, 13, 15) is selective thereby suggesting the likelihood that altered chemical composition of the homogenate from ethanol-fed rats artifically produces abnormal mitochondria, since such changes could hardly be expected to be selective.

Effect of Chronic Ethanol Consumption on Oxygen Uptake—The State 3 rates of oxygen consumption were comparable when control mitochondria oxidized palmitoyl-1-carnitine, palmitoyl-CoA, palmitate, oleate, or octanoate as the substrates (Table I). Although the rates in mitochondria from ethanol-fed animals were lower than in controls (see below), there were still no differences in rates between various substates. We previously found that the rates of oxygen consumption in State 4 were depressed 10 to 20% in mitochondria from ethanol fed rats, using NAD⁺-dependent substrates, succinate, a flavin-linked substrate, or ascorbate, which reduces cytochrome c (13). In State 4, the rate of oxygen consumption associated with the oxidation of all fatty acid substrates was also depressed 10 to 15% in mitochondria from ethanol-
The greater reduction in ADP-stimulated oxygen uptake (State 3) after ethanol feeding, compared to that in State 4, suggests an effect of chronic ethanol treatment on the coupling apparatus. In the presence of dinitrophenol, the rate of oxygen consumption associated with the oxidation of palmitoyl-1-carnitine, palmitoyl-CoA, and palmitoyl-l-carnitine in mitochondria from ethanol-fed rats (Table II). This decrease was similar to that observed in the absence of the uncoupler (State 4). Thus, the stimulation by dinitrophenol was not altered by ethanol feeding (Table II). This contrasts with the greater reduction in ADP-stimulated oxygen consumption (29 to 32%, Table I) and the decrease in the respiratory control ratio (Fig. 1) found after ethanol ingestion. Whereas ADP was as effective as dinitrophenol in stimulating oxygen consumption in control mitochondria, it was not as effective as dinitrophenol in stimulating oxygen consumption in mitochondria from ethanol-fed rats (compare Fig. 1 and Table II).

Chronic ethanol consumption apparently produces a defect in the respiratory chain, which is responsible for the slight decrease in fatty acid oxidation in State 4 or in the presence of an uncoupler (10 to 15%). Superimposed upon this effect is an inhibition of oxidative phosphorylation by ethanol feeding, which results in greater inhibition of ADP-stimulated respiration (30%).

Effect of Chronic Ethanol Consumption on CO2 Production—Oxygen consumption associated with the oxidation of long chain fatty acids is reduced to a greater extent by
ethanol consumption than that associated with the oxidation of medium chain fatty acids. It is also possible that ADP does not stimulate oxygen consumption in control mitochondria with octanoate to the same extent as with palmitate. However, it does stimulate oxygen uptake comparably with both substrates in mitochondria from ethanol-fed rats.

To corroborate these impressions, we studied the effect of ethanol feeding on CO₂ production from ¹⁴C-labeled palmitate and octanoate. As shown in Fig. 2, CO₂ production from palmitate was reduced 37% in mitochondria from ethanol-fed rats, a value comparable to the 32% decrease in State 3 oxygen consumption. CO₂ production from octanoate was reduced 20% by ethanol feeding, similar to the 17% decrease in State 3 oxygen consumption. By either technique, palmitate oxidation was more sensitive to depression by ethanol feeding than was octanoate oxidation.

Effect of Chronic Ethanol Consumption on Palmitoyl-CoA Synthetase and Carnitine Palmitoyltransferase—Factors which might play a role in the differential inhibition of long chain and medium chain fatty acids include the fatty acid synthetase of the outer membrane, which activates long chain, but not medium chain fatty acids (24, 25), and carnitine palmitoyltransferase, which transports long chain fatty acids into the mitochondria (26, 27). We, therefore, investigated the effects of chronic ethanol feeding on the activities of these two enzymes. Palmitoyl-CoA synthetase activity was not affected by ethanol feeding (specific activity (micromoles of hydroxylö acid formed per hour per mg of protein) of 3.7 ± 0.4 for control mitochondria and 3.5 ± 0.5 for mitochondria from ethanol-fed rats). By contrast, carnitine palmitoyltransferase activity was stimulated 30% (specific activity (nanomoles of CoA released per min per mg of protein) of 27 ± 1.4 for control mitochondria and 35 ± 4 for mitochondria from ethanol-fed rats, p < 0.05). Thus, the depression of total fatty acid oxidation by ethanol feeding cannot be explained by an effect on the activities of these enzymes.

Effect of Chronic Ethanol Consumption on Ketogenesis—It seemed possible that chronic ethanol consumption might favor a flow of acetyl-CoA, derived from β oxidation, into ketogenesis rather than to total oxidation to CO₂. Such a shift would cause CO₂ production and O₂ uptake to be reduced, but β oxidation of fatty acids would not necessarily be depressed. In other studies a redistribution of acetyl-CoA was suggested to explain the inhibition of palmitate oxidation to CO₂ by acetate (28). We, therefore, investigated the effects of chronic ethanol feeding on ketone body production by hepatic mitochondria. Ethanol feeding had no effect on the endogenous rate of ketone body production (40.88 ± 5.4 nmol of β-hydroxybutyrate and acetoacetate formed per 30 min per mg of protein for control mitochondria and 42.5 ± 3.5 for mitochondria from ethanol-fed rats). The addition of palmitoyl-1-carnitine or palmitate increased ketone body production about 4-fold. There was a slight increase in ketone body production in mitochondria from ethanol-fed rats (+9 to +15%), but this did not reach statistical significance. Similar results were obtained with lower concentrations of fatty acids, e.g. with 30 μM palmitoyl-1-carnitine, there was an 11% increase in ketogenesis after ethanol feeding.

Pande (29) has suggested that ketone body production in liver may be enhanced by the suppression of the citric acid cycle. We, therefore, investigated ketogenesis after inhibiting the activity of the citric acid cycle with fluorocitrate. With

### Table II

**Effect of chronic ethanol consumption on dinitrophenol stimulation of fatty acid oxidation**

| Substrate                          | Reaction | Oxygen uptake | Effect | p   |
|------------------------------------|----------|---------------|--------|-----|
|                                    |          | Control (nmol/min/mg protein) | Ethanol (nmol/min/mg protein) | %      | p    |
| Palmitoyl-1-carnitine (6)          | State 4  | 21.3 ± 2.77   | 18.10 ± 2.12 | -15   | < 0.05 |
|                                    | +DNP     | 76.0 ± 8.90   | 64.90 ± 6.10 | -15   | 0.05  |
|                                    | -DNP: -DNP | 3.47 ± 0.30   | 3.58 ± 0.13  | +3    | N.S.  |
| Palmitoyl-CoA (6)                  | State 4  | 19.60 ± 1.60  | 16.46 ± 2.10 | -16   | < 0.01 |
|                                    | +DNP     | 71.97 ± 6.90  | 60.70 ± 7.60  | -16   | 0.05  |
|                                    | -DNP: -DNP | 3.69 ± 0.46   | 3.70 ± 0.73   | 0     | N.S.  |
| Palmitate (6)                      | State 4  | 28.22 ± 2.52  | 23.80 ± 3.50  | -16   | < 0.01 |
|                                    | +DNP     | 71.06 ± 7.90  | 57.78 ± 1.80  | -19   | < 0.05 |
|                                    | -DNP: -DNP | 2.61 ± 0.57   | 2.52 ± 0.56   | -4    | N.S.  |

* N.S., not statistically significant.

### Fig. 2

Fig. 2. Effect of chronic ethanol ingestion on CO₂ production from labeled palmitate and octanoate. CO₂ was collected and counted as described under "Materials and Methods," with 75 μM ¹⁴C-labeled palmitate (about 900 cpm/nmol) or octanoate (about 1000 cpm/nmol) as the substrates. Number of pairs studied = 12 for palmitate and 10 for octanoate.
labeled palmitate and octanoate, 25 μM fluorocitrate inhibited CO₂ production 70 to 80% in mitochondria from ethanol-fed rats and 80 to 90% in controls. In the presence of fluorocitrate, there was no difference in the endogenous rate of ketone body production between control mitochondria and those from ethanol-fed rats (Fig. 3). However, compared to the rates in the absence of fluorocitrate, this endogenous rate increased 70% in control mitochondria and 79% in those from animals given ethanol, suggesting diversion of acetyl-CoA into ketogenesis, a more accessible pathway. Fluorocitrate was reported to increase ketogenesis and decrease citrate formation from palmitoyl carnitine and malate in isolated mitochondria (30). Upon adding palmitoyl-1-carnitine or palmitate, ketogenesis was increased about 3-fold in control mitochondria, and about 4-fold in mitochondria from ethanol-fed rats (Fig. 3). The total rates of ketogenesis were greater in the presence of fluorocitrate than in its absence, with both mitochondrial preparations. Both the total and net rates of ketone body production were greater in mitochondria from ethanol-fed rats than controls, with either substrate. Thus, in the presence of fluorocitrate, ketogenesis was stimulated after ethanol feeding. This may explain, in part, the increase in serum ketone bodies which occur after chronic ethanol feeding (31). The increase in ketone body production after ethanol ingestion may be due to increased activities of enzymes which participate in the formation of ketones or increased formation of acetyl-CoA via β-oxidation (or both). The increase in ketone body production (and formazan formation, see below) suggests that ethanol feeding does not impair β-oxidation of fatty acids to the level of acetyl-CoA.

**Oxidation of Fatty Acids in Presence of Artificial Electron Acceptors**—The ethanol-induced decrease in fatty acid oxidation under State 4 conditions or in the presence of uncoupler points to some impairment of the respiratory chain. The additional reduction in ADP-stimulated fatty acid oxidation suggests impairment of coupled phosphorylation (13). Others have suggested that ADP entry into the mitochondria may be compromised (23). To verify the results described above, and to eliminate the influence of changes in the respiratory chain, we studied fatty acid oxidation under anaerobic conditions, with the use of artificial electron acceptors to reoxidize NADH.

Initial studies were concerned with characterization of the system in mitochondria from rats fed commercial chow. This diet contains 9 to 10% of total calories as fat, whereas the liquid diet contains 35%. Formazan production was linear for 60 min, and was proportional to concentration between 0.2 and 2 mg of mitochondrial protein per ml. At higher protein concentrations, it was difficult to extract the formazan from the protein precipitate. Changing the concentrations of methylene blue, triphenyltetrazolium, NAD⁺, or ATP had no effect on the reaction. There were no significant differences in formazan production in mitochondria incubated under hypotonic or isotonic conditions, suggesting that the dyes had unrestricted access to the sites of β-oxidation and citric acid cycle activity. The addition of 5 mM citrate increased formazan production from 12.9 to 26.2 nmol/hour/mg of protein, producing reducing equivalents via citric acid cycle activity. An inhibitor of citrate transport, 10 mM 1,2,3-benzene tricarboxylic acid (32) blocked the citrate-induced increase (rate = 14.3), which indicates that the integrity of the membranes and receptor sites was preserved. Table III shows that the rate of formazan production is increased upon adding fatty acids to the system. α-Bromopalmitate, a competitive antagonist of palmitate metabolism (33), reduced the rate of formazan production in the presence of palmitate by 39%. The extent of inhibition may even be greater since α-bromopalmitate itself may serve as a substrate (Table III). The above studies show that the dyes effectively accept electrons from reducing equivalents produced by citric acid cycle activity or fatty acid oxidation (or both).

Mitochondria isolated from rats fed ethanol for 24 days displayed a slightly higher rate of endogenous formazan production than their pair-fed controls (Table IV). Upon the addition of palmitate the total rate, as well as the net rate of formazan production, was higher in mitochondria from ethanol-fed rats. Comparable results were obtained in mitochondria from two pairs of rats, with the use of octanoate or oleate as substrates. By contrast, there were no changes in formazan production after acute administration of ethanol (6 g/kg; 34.65 nmol of formazan/hour/mg for controls oxidizing palmitate, 34.98 for acute ethanol). Thus in the absence of a functional respiratory chain, mitochondria from ethanol-fed rats show a higher rate of formazan production, either because of increased β-oxidation, or increased activity of the citric acid cycle. To dissociate these two possibilities, we studied formazan production in the presence of fluorocitrate, which inhibits

![Fig. 3. Effect of chronic ethanol ingestion on ketone body production in the presence of fluorocitrate. Keton body production was assayed as described under “Materials and Methods” in the presence of 25 μM fluorocitrate. The endogenous rate refers to the rate in the absence of externally added fatty acid. The net rate is the total rate minus the endogenous rate. The concentration of fatty acids was 100 μM. Number of pairs studied = 8 for palmitoyl-1-carnitine and 6 for palmitate.](http://www.jbc.org/)

TABLE III

| Addition                    | Fornazan production | Effect  |
|-----------------------------|---------------------|---------|
|                             | Total rate          | Net rate|         |
|                             | nmol/hour/mg protein|         | %       |
| None                        | 12.9 ± 1.98         |         | +72     |
| Palmitate                   | 34.98 ± 2.64        | 22.11 ± 3.30 | +169   |
| Oleate                      | 34.65 ± 2.31        | 21.78 ± 2.64 | +169   |
| Octanoate                   | 26.40 ± 2.64        | 13.53 ± 2.97 | +105   |
| α-Bromopalmitate            | 17.49 ± 2.97        | 4.62 ± 2.64 | +36     |
| Palmitate + α-bromopalmitate| 26.40 ± 1.65        | 13.53 ± 1.98 | -104   |
the metabolism of two carbon fragments via the citric acid cycle. Under anaerobic conditions in the presence of fluorocitrate, formazan production upon the addition of palmitate presumably is a measure of $\beta$ oxidation. Fluorocitrate depressed the endogenous rate in the controls by 41%, but only by 23% in mitochondria from ethanol-fed rats (Table IV). With palmitate as the substrate, the total rate of formazan production was increased 39% by ethanol ingestion, compared to pair-fed controls. The net rate was also 28% greater in mitochondria from ethanol-treated animals (Table IV). Therefore, $\beta$ oxidation does not appear to be decreased by chronic ethanol feeding since formazan production and ketogenesis were actually increased by ethanol feeding.

Effect of Chronic Ethanol Consumption on Oxygen Uptake in Presence of Fluorocitrate—We previously found that acetaldehyde inhibits oxygen consumption in the presence and absence of fluorocitrate, indicating inhibition of the oxygen uptake which arises both from $\beta$ oxidation and from citric acid cycle activity (59a). In the presence of fluorocitrate oxygen consumption reflects $\beta$ oxidation of fatty acids to acetyl-CoA. Since $\beta$ oxidation in the presence of fluorocitrate is stimulated by chronic ethanol consumption, oxygen uptake under these conditions should also be stimulated. On the other hand, a lack of stimulation of oxygen uptake would imply inhibitory effects on the respiratory phosphorylation chain. In the presence of fluorocitrate, oxygen uptake associated with the oxidation of fatty acids was slightly decreased in mitochondria from ethanol-fed rats (Table V). This suggests impairment of the respiratory phosphorylation chain by chronic ethanol consumption. However, the relative decrease in oxygen consumption was less than that found in the absence of fluorocitrate (compare with Table I). The greater inhibition of oxygen uptake in the absence of fluorocitrate suggests an effect of chronic ethanol feeding on citric acid cycle activity.

DISCUSSION

In this study fatty acid oxidation by isolated hepatic mitochondria was depressed after chronic ethanol feeding, suggesting that persistent changes in mitochondrial functions, in addition to the effects produced by metabolism of ethanol, may play a role in the production of fatty liver. The decreases in oxygen uptake and CO$_2$ production from fatty acids do not by themselves prove that fatty acid utilization is impaired, because acetyl-CoA derived from $\beta$ oxidation may be diverted to other pathways, e.g., ketogenesis or fatty acid elongation, rather than to oxidation via the citric acid cycle. That such a diversion may occur is indicated by the demonstration that acetate depresses oxygen uptake and CO$_2$ production from palmitate, whereas ketogenesis is stimulated (28). We, therefore, examined some of the factors which may participate in the depression of fatty acid oxidation after ethanol feeding, including activation and translocation of fatty acids, $\beta$ oxidation, ketone body production, and the activities of the citric acid cycle and the respiratory phosphorylating system.

We previously found that the State 3 oxidation of several NAD$^+$-dependent substrates was depressed after ethanol feeding much more than uncoupler-stimulated or State 4 oxygen consumption. These data suggested that in addition to a defect in the respiratory chain produced by chronic ethanol consumption there is also an inhibitory effect on oxidative phosphorylation (13). In this study, similar results were obtained in studies of fatty acid oxidation. Thus, State 4 or uncoupler-stimulated oxygen uptake was decreased 10 to 15% by ethanol feeding, whereas the State 3 rate was reduced about 30%. That oxidative phosphorylation is impaired is further suggested by the decrease in the respiratory control ratio, whereas the stimulation of fatty acid oxidation by dinitrophenol was not altered by ethanol feeding. In the presence of fluorocitrate, oxygen uptake associated with fatty acid oxidation was also depressed after ethanol feeding. Thus in the absence of activity of the citric acid cycle, the inhibitory effect of ethanol feeding on oxidative phosphorylation results in a net inhibition of oxygen uptake. There are additional reports indicating that chronic ethanol consumption decreases oxygen consumption by hepatic mitochondria (23, 34, 35).

The decrease in CO$_2$ production from labeled palmitate or octanoate suggests the possibility of an inhibitory effect of chronic ethanol feeding on the activity of the citric acid cycle. The ethanol-induced decrease in oxygen uptake was greater in the presence of intact citric acid cycle activity than when fluorocitrate inhibited the cycle. Thus, inhibition of oxygen uptake by ethanol feeding involves depression of oxygen consumption which arises from citric acid cycle activity. The fact that formazan production and ketogenesis are stimulated, but total oxidation of fatty acids is depressed, suggests that acetyl-CoA is diverted from the citric acid cycle to other pathways, e.g., ketogenesis. In preliminary experiments we found that CO$_2$ production from various $^{14}$C-labeled citric acid cycle intermediates was decreased 15 to 25% in mitochondria from ethanol-fed rats (34). In liver cells metabolizing ethanol, Ontko (36) suggested that inhibition of the citric acid cycle

| Substrate | Oxygen uptake | Control | Ethanol | Effect | $p$ |
|-----------|---------------|---------|---------|--------|-----|
| Palmitoyl-carnitine | 62.86 ± 5.80 | 52.0 ± 8.96 | -17 | 0.05 < $p$ < 0.10 |
| Palmitate (5) | 60.16 ± 4.40 | 51.04 ± 5.40 | -15 | < 0.05 |
| Oleate (6) | 58.30 ± 2.72 | 49.29 ± 6.00 | -16 | < 0.05 |
occurred at the level of α-ketoglutarate dehydrogenase, with a minor site of inhibition at the span beyond succinate. In the perfused liver, sites of inhibition during ethanol oxidation were identified at the citrate synthetase and isocitrate dehydrogenase steps (37). The inhibitions observed in those studies involve active metabolism of ethanol, with a consequent alteration of the oxidation-reduction state of the mitochondria. Further studies are in progress to study directly the possibility that persistent changes caused by chronic ethanol feeding may also alter citric acid cycle activity, independent of the changes caused by the oxidation of ethanol.

In these studies we did not examine the possible utilization of acetyl-CoA in fatty acid elongation, since the cofactors necessary for significant rates of elongation were not present. In the absence of NADPH, elongation is a sluggish process (less than 0.01 nmol of acetyl-CoA incorporated per min per mg (38, 39)) compared to the rates of ketogenesis or total oxidation to CO₂. The finding that the 20:4 to 18:2 fatty acid ratio is depressed after ethanol feeding (40, 41) suggests that, if anything, fatty acid elongation may be decreased by ethanol feeding.

Ethanol feeding induced a slight increase in ketone body production and formazan formation, which was augmented considerably in the presence of fluorocitrate. These data suggest that mitochondrial β oxidation to the level of acetyl-CoA is increased, rather than decreased by chronic ethanol ingestion. This is consistent with the increased blood levels of ketone bodies and increased acetoacetate formation by liver slices from rats chronically fed ethanol (31). In view of the fact that formazan production and ketogenesis are increased rather than decreased in mitochondria from ethanol-fed rats, the decrease in CO₂ production from fatty acids may be caused either by depression of citric acid cycle activity or by impaired oxidative phosphorylation.

Mitochondria from rats fed a high fat diet (35% of total calories) display higher endogenous and total rates of β oxidation (formazan production) than mitochondria from rats fed a chow diet in which fat provides 9 to 10% of total calories. β oxidation may be induced by a high fat diet and is further enhanced by ethanol feeding. The increase in carnitine palmitoyltransferase activity after ethanol feeding may contribute to the increased rate of β oxidation, by transporting fatty acids to the site of β oxidation. However, for reasons described below, it is unlikely that this enzyme normally is rate-limiting for fatty acid oxidation. It is possible that the increase in β oxidation and ketone body production provides a mechanism for disposing of excess fatty acids.

The decreased rate of fatty acid oxidation cannot be attributed to the presence of ethanol in the tissue, since the washing procedures would have removed any contaminating ethanol. Furthermore, addition of ethanol in vitro had no effect on fatty acid oxidation. The addition of ATP, CoA, or NAD⁺ did not prevent the reduction in fatty acid oxidation after ethanol feeding, suggesting that nucleotide or cofactor depletion is not a factor. Acetaldehyde (0.6 to 3.0 mM) inhibited the oxidation of fatty acids by rat liver mitochondria, as assayed by oxygen consumption and CO₂ production (33a). Oxygen uptake was depressed by acetaldehyde in the presence and absence of fluorocitrate. ADP-stimulated oxygen uptake was more sensitive to inhibition by acetaldehyde than was uncoupler-stimulated oxygen uptake, and, as a consequence, acetaldehyde depressed the respiratory control ratio associated with the oxidation of fatty acids. Thus, there are many similarities in the effects of chronic ethanol feeding and acetaldehyde on mitochondrial fatty acid oxidation. Acetate (1 to 3 mM) also depressed oxygen uptake and CO₂ production from palmitate, whereas it stimulated ketogenesis from palmitoyl-CoA (28). Thus it is possible that metabolites of ethanol oxidation, as well as persisting structural damage to the mitochondria, may account for the impairment of fatty acid oxidation in mitochondria from chronically intoxicated rats.

Chronic ethanol consumption has differential effects on the oxidation of long chain and medium chain fatty acids; oxygen consumption and CO₂ production were depressed to a greater extent with palmitate than with octanoate. This differential inhibition apparently does not involve activation of palmitate to palmitoyl-CoA or transfer of palmitate into the mitochondria as the carnitine ester, since palmitoyl-CoA synthesis activity was not affected by ethanol feeding, and carnitine palmitoyltransferase activity was increased. In addition, octanoate oxidation was also depressed to some extent by ethanol feeding; octanoate is activated in the inner membrane matrix of the mitochondria (25) and does not require carnitine for transport into the mitochondria (26). The differential effect of chronic ethanol feeding on long chain and medium chain fatty acid oxidation is similar to that observed for acetaldehyde and acetate, since octanoate oxidation was less sensitive to inhibition by these metabolites of ethanol oxidation than was palmitate oxidation (28, 33a). Replacement of dietary long chain fatty acids by medium chain fatty acids reduces the hepatic triglyceride accumulation found after ethanol consumption (42). This has been attributed to the propensity of octanoate to undergo oxidation rather than esterification (42). Another contributing factor may be the fact that the oxidation of octanoate is less sensitive to ethanol feeding (or acetaldehyde or acetate (or both)) than that of palmitate.

It has been suggested that the fatty acyl synthetase (43, 44) or the carnitine palmitoyltransferase (45, 46) reactions may be rate-limiting steps in fatty acid oxidation. Pande (29), however, showed that in liver mitochondria a rate-limiting factor in acetyl-CoA production from palmitate is the capacity of the electron transport-oxidative phosphorylation system. The comparable rates of oxygen uptake when palmitate, palmitoyl-CoA, or palmitoyl-1-carnitine are employed as substrates suggest that activation or translocation of fatty acids into the mitochondria are not rate-limiting for fatty acid oxidation. The activity of carnitine palmitoyltransferase was at least 5-fold greater than the rate of palmitate utilization. Furthermore, chronic ethanol consumption depressed fatty acid oxidation, although carnitine palmitoyltransferase activity was stimulated. The greater reduction by ethanol feeding of State 3 oxygen consumption than of State 4 or uncoupler-stimulated oxygen uptake with fatty acids as the substrates, suggests that part of the mechanism behind the inhibition of fatty acid oxidation may relate to the rate-limiting capacity of the electron transport-oxidative phosphorylation system.

To verify the results obtained by conventional methods of assaying fatty acid oxidation, we adapted the method of Mii and Green (20) to assess the rate of β oxidation by intact mitochondria. Although this method uses unphysiological conditions, such as anaerobiosis and artificial electron acceptors, the data parallel those obtained by measuring ketone body production. Formazan production was increased by substrates which provide reducing equivalents via the citric acid cycle (citrate) or from β oxidation (fatty acids). This increase was prevented by inhibitors of citrate transport.
(benzene tricarboxylate) or palmitate transport (α-bromopalmitate). In this system, α-bromopalmitate, which antagonized the increase in formazan production produced upon addition of palmitate, could also serve as a substrate for formazan production. α-Bromopalmitate could also serve as a substrate for the reconstituted fatty acid shuttle (47). These results imply that α-bromopalmitate can enter the mitochondria, bypassing the soluble carnitine palmitoyltransferase A (48, 49) and serving as a substrate for the membrane-bound carnitine palmitoyltransferase B. Indeed West et al. (48) found only the soluble enzyme to be inhibited by α-bromopalmitoyl-CoA, whereas the membrane-bound enzyme could generate α-bromopalmitoyl-CoA within the matrix, from externally added α-bromopalmitate (50).

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