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# Effects of ethanol on the metabolism of free fatty acids in isolated liver cells

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**Abstract** Ethanol inhibited the oxidation and enhanced the esterification of albumin-bound [1-$^{14}$C]palmitate incubated with isolated rat liver cells. Ethanol decreased the conversion of [1-$^{14}$C]palmitate to $^{14}CO_2$ and $^{14}$C-labeled ketone bodies and enhanced the incorporation of [1-$^{14}$C]palmitate into glycerolipids, especially triglyceride; cholesteryl ester synthesis was unaffected. The half-maximal effective ethanol concentration for each of these processes was 6–10 $\mu$g/ml and a maximum effect was produced by about 50 $\mu$g/ml. Ethanol oxidation was required for each of these alterations, since the effects were completely abolished by pyrazole. The energy obtainable from ethanol oxidation was in excess of the energy deficit from decreased fatty acid oxidation. However, ethanol did not affect $O_2$ consumption, indicating that ethanol oxidation replaced the oxidation of both fatty acids and other substrates. Ethanol inhibited the citric acid cycle in the intact liver cells by 20–30%. The major site of inhibition was $\alpha$-ketoglutarate oxidation. Results suggest that ethanol inhibited $\alpha$-ketoglutarate dehydrogenase in the mitochondria of hepatocytes by elevating the mitochondrial NADH:NAD ratio. A minor site of inhibition of ethanol oxidation was detected between succinate and citrate. It is suggested that ethanol inhibits fatty acid oxidation in hepatocytes by competitive substrate oxidation, resulting in an increased availability of long-chain free fatty acids; this thereby enhances esterification, leading to accumulation of liver triglyceride.

**Supplementary key words** fatty acid oxidation · ketone bodies · esterification · triglycerides · phospholipids · citric acid cycle · $\alpha$-ketoglutarate dehydrogenase · isocitrate dehydrogenase · competitive substrate oxidation · pyrazole

Since Mallov and Bloch (1) observed that acute ethanol administration leads to the accumulation of triglyceride (2) in the liver, there have been numerous investigations on the mechanism of this effect, as recently reviewed by Lieber, Rubin, and DeCarli (3). Ethanol also causes an elevation of plasma triglycerides in both fed and fasting states (3–5). Plasma triglycerides are derived mainly from the liver in the postabsorptive state (6), suggesting that the elevation of both liver and plasma triglyceride concentrations results substantially from the same effect of ethanol on lipid metabolism. Liver triglyceride accumulation and enhanced plasma triglyceride formation, reported after ethanol administration in fasting subjects (7), supports this concept.

Acute ethanol intake leads to the deposition of liver triglycerides with a fatty acid composition resembling that of adipose tissue (8–10). This clearly implicates plasma FFA as the predominant precursors of the liver triglycerides after a single dose of alcohol (even though the plasma FFA level is unaltered or decreased by ethanol in less than massive doses [3, 11, 12]), since it is well established that circulating FFA is derived from the adipose tissue (13).

An ethanol-induced decrease in fatty acid oxidation in the liver has been suggested as a possible basis for the development of the fatty liver (14, 15). This concept was based on the measurement of the complete oxidation of fatty acids to $CO_2$. Although a significant effect of ethanol on citric acid cycle activity in the liver has not been found in every case (16), sufficient evidence has accumulated from several laboratories to establish that ethanol markedly inhibits the activity of the citric acid cycle in this tissue (7, 14, 15, 17–20). Ketone bodies are major end products of fatty acid oxidation in the liver. Ethanol has been reported to increase (17, 19, 21, 22) and decrease (7, 16, 23) ketogenesis. These differences remain unexplained.

Abbreviations: FFA, free fatty acid(s).

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Several years ago Nikkila and Ojala (24) observed that acute ethanol administration promotes the esterification of circulating FFA in the liver. Incorporation of palmitate into triglyceride was increased, whereas its incorporation into phospholipids was unaffected. Others have reported enhanced synthesis of triglycerides from long-chain fatty acids by liver homogenates (25) and liver slices (9) from ethanol-treated rats. Although the ethanol-enhanced incorporation of long-chain fatty acids into liver lipids has been repeatedly observed (7, 16, 26), its molecular basis remains obscure.

The underlying mechanisms of these effects of ethanol on the metabolism of FFA in liver have been investigated in the present study by measuring each of the three major metabolic reactions of palmitic acid, namely, esterification, partial oxidation to ketone bodies, and complete oxidation to $CO_2$, in isolated liver cells. The ethanol concentration-dependence of these effects was determined. The influence of ethanol on the formation of phospholipids, diglycerides, triglycerides, and cholesteryl esters was also measured. In addition, the sites of interaction of ethanol oxidation on the citric acid cycle have been determined in the intact liver cells.

## METHODS

### Isolation and incubation of liver cells

Liver cells were isolated from rats fasted 24 hr by the procedure of Berry and Friend (27) as previously described (28). The isolated cells were suspended in calcium-, glucose-, and bicarbonate-free Hanks solution containing 10 mM sodium phosphate buffer, pH 7.4. 1 ml of isolated liver cells was incubated together with 1 ml of 3% albumin containing 0.75 mM [1-$^{14}$C]palmitate dissolved in the same medium. Prior to preparation of the albumin-bound palmitate solution, the albumin (crystalline bovine serum albumin from Pentex, Kankakee, Ill.) was charcoal-treated by the method of Chen (29) to remove free fatty acids and other impurities (30). Incubations in duplicate were carried out at 37°C in Erlenmeyer flasks with center wells containing 0.2 ml of 10% KOH and filter paper for $^{14}CO_2$ and $^{14}$C-labeled acetoacetate analyses as previously described (28). In other Erlenmeyer flasks, cells were incubated in duplicate for lipid extraction with 20 vol of chloroform–methanol 2:1 (v/v) (28). Additional duplicate incubations were carried out for analyses of acetoacetate (31) and $\beta$-hydroxybutyrate (32) as previously described (28).

### Thin-layer chromatography

The chloroform–methanol extracts were filtered and washed according to the procedure of Folch, Lees, and Sloane Stanley (33). The lipids in the extract were then separated on silica gel G containing Ultraphor (34), using a solvent system of hexane–diethyl ether–glacial acetic acid 80:20:1 (v/v/v). Bands were identified with ultraviolet light.

### Liquid scintillation counting

The $^{14}CO_2$ and $^{14}$C-labeled acetoacetate were counted as previously described (28). The radioactivity in phospholipids, diglycerides, FFA, triglycerides, and cholesteryl esters was counted after scraping the silica gel areas containing these lipids into counting vials and adding scintillation fluid containing Cab-O-Sil as described previously (35).

### Decarboxylation of long-chain fatty acids

The radioactivity in the carboxyl carbon of the precursor [1-$^{14}$C]palmitate was compared with the carboxyl carbon radioactivity in the long-chain fatty acids in lipid ester products by the method previously described (28).

### Oxygen consumption

The rate of oxygen consumption of the isolated liver cells was measured polarographically with a Clark electrode in a 1.0-ml closed vessel containing 5–10 mg of cells (dry wt) in suspension medium.

### Ethanol analysis

Ethanol utilization was measured enzymatically in perchloric acid extracts of liver cells (36). It was found necessary to incubate the cells in capped flasks to prevent loss of ethanol by evaporation.

### Quality of the liver cells

The isolated liver cells used in these studies were structurally and metabolically intact. Over 90% of the cells in each preparation excluded trypan blue. Further evidence regarding the quality of these cells included a constant rate of gluconeogenesis and a very low amount of leakage of the cytoplasmic enzyme lactate dehydrogenase into the medium (28). The cells remained intact throughout the incubation period.

### Rate of product formation and effect of cell concentration

The rate of formation of $^{14}CO_2$, $^{14}$C-labeled ketone bodies, and $^{14}$C-labeled fatty acid esters from [1-$^{14}$C]-palmitate by isolated liver cells under the conditions employed in this investigation was almost completely constant throughout the 30-min incubation period both in the presence and absence of ethanol. Analysis of product formation at 10, 20, and 30 min in separate experiments showed that the quantity of $^{14}$C in all three products at 20 min was twice that at 10 min. Slightly slower incorporation was sometimes observed during the last 10 min, notably when the cells were incubated in concen-



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trations exceeding 15 mg (dry wt)/ml, owing to precursor depletion. When this occurred, the same minor decrease in rate was observed in the presence and absence of ethanol. In the four experiments summarized in Table 1, cells were incubated at concentrations of 11.3, 15.7, 14.8, and 20.7 mg (dry wt)/ml. It was also shown in separate experiments that the formation of the products was directly proportional to the cell concentration, when incubated for 30 min, in a range from 7.5 mg to 15 mg (dry wt)/ml. Only slight, if any, deviation from proportionality occurred at 20 mg (dry wt)/ml but marked deviation was observed at 30 mg (dry wt)/ml, owing to depletion of albumin-bound palmitate.

## RESULTS

Ketone bodies were the major products of palmitic acid metabolism in the liver cells isolated from fasted rats (Table 1). A considerable quantity of the added palmitate was esterified, and a smaller quantity was oxidized to $CO_2$. Ethanol had significant and reproducible effects on all three processes. Ketogenesis and $CO_2$ formation were inhibited, and esterification was markedly enhanced. Fatty acid esters were the major conversion product in the presence of ethanol. The average net increase in esterification was 62.1 nmoles/20 mg dry wt of liver cells/30 min (Table 1). Ketogenesis and $CO_2$ formation decreased by 39.3 and 6.7 nmoles/20 mg dry wt/30 min, respectively. The total increase in fatty acid esters was 16.1 nmoles greater than the total decrease in oxidation products. This difference is probably due to an appreciable amount of radioactivity at the end of the incubation in the form of citric acid cycle and other intermediates produced from acetyl CoA derived from the $\beta$-oxidation of [1-$^{14}$C]palmitate. The formation of these intermediates should be decreased consequent to the ethanol-induced inhibition of the $\beta$-oxidation of palmitate, and a corresponding reduction in this $^{14}$C pool could account for this difference. Under the conditions of the incubation (see Table 1 footnote), 25–50% of the added palmitate was utilized during the incubation period.

The observed ethanol-induced increase in the radioactivity of lipid esters following incubation with [1-$^{14}$C]-palmitate (Table 1) could conceivably result from *1)* increased direct esterification, *2)* increased chain elongation utilizing [1-$^{14}$C]acetyl CoA, or *3)* enhanced de novo synthesis from [1-$^{14}$C]acetyl CoA. These possible routes have been discussed previously (28). The carboxyl radioactivity in the fatty acids of the triglycerides and phospholipids formed from [1-$^{14}$C]palmitate during incubation of liver cells with and without ethanol was therefore measured. Between 90 and 94% of the triglyceride and phospholipid radioactivity extracted from the cells incubated both with and without ethanol was recovered in the carboxyl carbon of the fatty acids. Similarly, 90–95% of the radioactivity of the [1-$^{14}$C]palmitate precursor was recovered in the carboxyl carbon with this procedure (see Methods). Therefore, carbon recycling via chain elongation or de novo synthesis does not contribute appreciably to the radioactivity found in the triglycerides and phospholipids. Ethanol therefore enhanced the direct esterification of [1-$^{14}$C]palmitate.

Even though ethanol decreased the conversion of added palmitate to ketone bodies, total ketogenesis was not appreciably affected (Table 2). It is likely that ethanol was in part converted to acetoacetate. This would account for the undiminished total ketogenesis and the decreased ketone body specific activity. Ethanol increased

TABLE 1. Effects of ethanol on utilization of palmitic acid by isolated liver cells

| Conversion Product | Palmitate Utilization | | | | |
|---|---|---|---|---|---|
| | Control | Ethanol (1 mg/ml) | % of Control | Difference | $P^a$ |
| | *nmoles*[b] | *nmoles*[b] | | *nmoles*[b] | |
| Esterified fatty acids | 65.9[c] | 128.0 | 196.0 | +62.1 | |
| | ±4.2 | ±2.9 | ±11.8 | ±4.3 | <0.001 |
| Carbon dioxide | 16.1 | 9.4 | 59.2 | −6.7 | |
| | ±1.2 | ±0.3 | ±3.8 | ±1.1 | <0.005 |
| Total ketone bodies | 158.8 | 124.5 | 78.8 | −34.3 | |
| | ±5.4 | ±3.2 | ±4.3 | ±7.8 | <0.01 |

In each of the experiments (n = 4), 1.0 ml of liver cells (isolated from rats fasted 24 hr) in suspension medium and 1.0 ml of 3% albumin–0.75 mM [1-$^{14}$C]palmitate in suspension medium were incubated for 30 min at 37°C. The added [1-$^{14}$C]palmitate in the four experiments contained 745,840 dpm, 775,650 dpm, 769,950 dpm, and 599,600 dpm, and the flasks contained 22.5, 31.4, 29.5, and 41.3 mg (dry wt) of liver cells, respectively. The percentage of added palmitate utilized, as measured by the quantity of $^{14}$C-labeled FFA recovered at the end of the incubation, was 46–75. This variation resulted largely from the differences in cell concentration.

[a] Determined by Student's *t* test for paired observations.

[b] nmoles of palmitate converted to the product indicated/20 mg (dry wt)/30 min.

[c] Values are means ± SE.



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TABLE 2. Effects of ethanol on ketogenesis by isolated liver cells

| | Control | Ethanol (1 mg/ml) | % of Control | Difference | P[a] |
|---|---|---|---|---|---|
| Total ketone bodies, μg[b] | 133.2[c] | 126.3 | 96.5 | +6.9 | |
| | ±19.3 | ±14.3 | ±5.5 | ±7.5 | NS |
| Ketone body specific activity, dpm/μg C | 2560 | 2100 | 82.0 | −473 | |
| | ±342 | ±276 | ±4.3 | ±106 | <0.01 |
| β-Hydroxybutyrate: acetoacetate ratio | 0.42 | 0.71 | 171.3 | −0.30 | |
| | ±0.04 | ±0.08 | ±16.9 | ±0.06 | <0.01 |

These results were obtained in the same four experiments described in Table 1.
[a] Determined by use of Student's $t$ test for paired observations.
[b] μg/20 mg dry wt/30 min.
[c] Values are means ± SE.

TABLE 3. Effects of ethanol on esterification of palmitic acid by isolated liver cells

| | Quantity of Palmitate Esterified | | | | |
|---|---|---|---|---|---|
| | Control | Ethanol (1 mg/ml) | % of Control | Difference | P[a] |
| | *nmoles*[b] | *nmoles*[b] | | *nmoles*[b] | |
| Triglyceride | 27.1[c] | 64.3 | 239.7 | +37.2 | |
| | ±2.6 | ±3.9 | ±10.8 | ±2.1 | <0.001 |
| Phospholipid | 31.2 | 49.6 | 160.3 | +18.4 | |
| | ±1.5 | ±1.2 | ±10.7 | ±2.4 | <0.005 |
| Diglyceride | 4.8 | 11.1 | 275.4 | +6.3 | |
| | ±1.0 | ±0.7 | ±68.1 | ±1.1 | <0.005 |
| Cholesteryl ester | 2.9 | 3.1 | 109.1 | +0.2 | |
| | ±0.4 | ±0.4 | ±4.7 | ±0.1 | NS |

These data were obtained in the same four experiments described in Table 1.
[a] Determined by use of Student's $t$ test for paired observations.
[b] nmoles/20 mg (dry wt)/30 min.
[c] Values are means ± SE.

the β-hydroxybutyrate:acetoacetate ratio, as previously observed (17, 19).

The ethanol-induced increase in palmitate esterification was found to result from enhanced formation of diglycerides, triglycerides, and phospholipids (Table 3). Esterification of palmitate to form cholesteryl esters was not affected. Formation of glycerolipids was specifically elevated. Enhanced triglyceride formation accounted for 60% of the total increase in esterification, whereas elevated phospholipid and diglyceride formation accounted for 30% and 10% of this increase, respectively.

The concentration-dependence of these effects of ethanol on palmitate esterification and oxidation to $CO_2$ and ketone bodies was determined (Fig. 1). The half-maximal effective ethanol concentrations for these conversions were between 6 and 10 μg/ml. An ethanol concentration of about 50 μg/ml produced a maximum effect on each of these main pathways of palmitate utilization.

Half-maximal effects of ethanol on phospholipid, diglyceride, and triglyceride formation from palmitate occurred at about 4, 15, and 7 μg of ethanol/ml, respectively (Fig. 2). Maximal effects on the conversion of palmitate to each of these glycerolipids was observed at an ethanol concentration of about 50 μg/ml. In this experiment, phospholipid, diglyceride, and triglyceride formation from palmitate was maximally increased 48%, 191%, and 126%, respectively, by ethanol. The increase in these esterified products accounted, respectively, for 25%, 11%, and 64% of the total ethanol-induced increase in glycerolipid formation.

The effects of ethanol in decreasing the oxidation of [1-$^{14}$C]palmitate to $^{14}CO_2$ and $^{14}$C-labeled ketone bodies and in increasing the formation of $^{14}$C-labeled fatty acid esters were completely abolished by preincubation of the isolated liver cells for 10 min with 1 mM pyrazole (Table 4).

To investigate the citric acid cycle reactions inhibited by ethanol in the intact cells (Table 1 and Fig. 1), effects of ethanol on the oxidation of various labeled compounds to $^{14}CO_2$ were measured (Table 5). The conversions of [1-$^{14}$C]acetate and [1-$^{14}$C]palmitate to $^{14}CO_2$ were both inhibited 40–45% by ethanol at a concentration of 1 mg/ml. [6-$^{14}$C]Citrate oxidation to $^{14}CO_2$ was unaffected, whereas $^{14}CO_2$ formation from [1,4-$^{14}$C]succinate, [1-$^{14}$C]-glutamate, and α-[1-$^{14}$C]ketoglutarate was decreased



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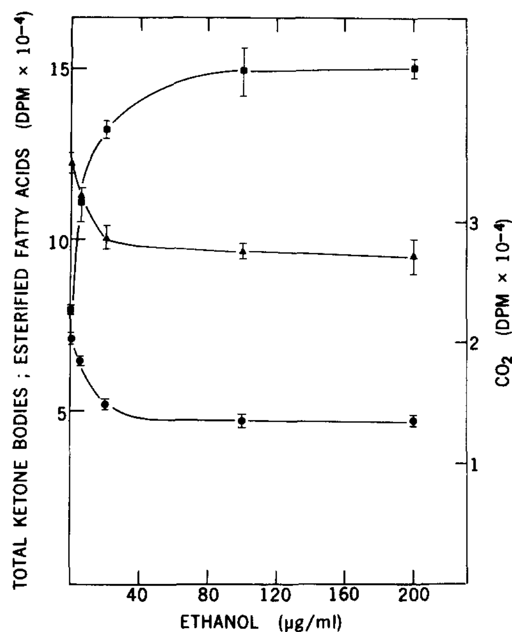


Fig. 1. Effect of ethanol concentration on the oxidation and esterification of [1-$^{14}$C]palmitate in isolated liver cells. Isolated liver cells (1 ml, 21.5 mg dry wt) from a fasted rat were incubated with 1 ml of 3% albumin–0.75 mm [1-$^{14}$C]palmitate (599,600 dpm) (see Methods) for 30 min at 37°C in the presence of 0, 5, 20, 100, and 200 μg of ethanol/ml. ■, total $^{14}$C-labeled lipid esters; ▲, total $^{14}$C-labeled ketone bodies; ●, $^{14}CO_2$.

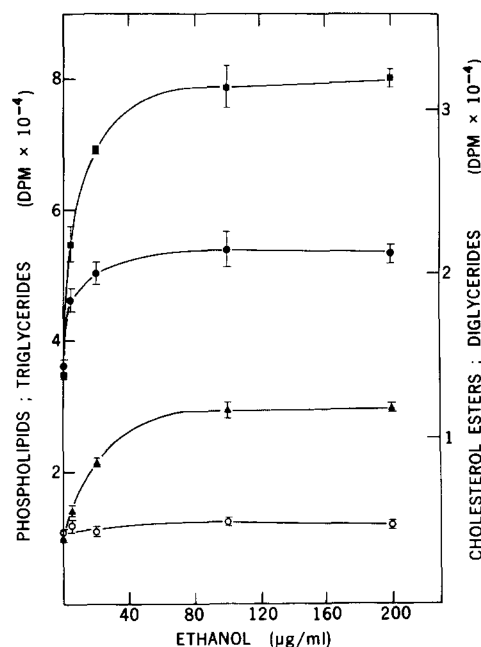


Fig. 2. Effect of ethanol concentration on the formation of various lipid esters from [1-$^{14}$C]palmitate. The total lipids in the experiment described in Fig. 1 were separated by thin-layer chromatography. ■, $^{14}$C-labeled triglycerides; ●, $^{14}$C-labeled phospholipids; ▲, $^{14}$C-labeled diglycerides; ○, $^{14}$C-labeled cholesteryl esters.

15%, 23%, and 24%, respectively (Table 5). The inhibition of the conversion of [1,4-$^{14}$C]succinate to $^{14}CO_2$ by ethanol was greater than half that of [1-$^{14}$C]glutamate and α-[1-$^{14}$C]ketoglutarate. It should be noted that this relationship was highly reproducible. Even though ethanol markedly inhibited the citric acid cycle, the rate of oxygen consumption of the cells, measured with the oxygen electrode, was unaltered. This indicates substitution of ethanol oxidation for the oxidation of other substrates.

TABLE 4. Prevention by pyrazole of ethanol-induced alterations in fatty acid oxidation and esterification

| | Conversion of [1-$^{14}$C]palmitate to: | | |
|---|---|---|---|
| | $CO_2$ | Total Ketone Bodies | Fatty Acid Esters |
| | | *nmoles*[a] | |
| Control | 14.8 | 180 | 76 |
| Pyrazole, 1 mm | 14.6 | 182 | 71 |
| Ethanol, 0.1 mg/ml | 10.4 | 153 | 124 |
| Ethanol, 0.1 mg/ml, + pyrazole, 1 mm | 14.3 | 181 | 75 |

Isolated liver cells, 29.3 mg (dry wt) in 1.0 ml of suspension medium, were incubated for 10 min at 37°C with and without 1 mm pyrazole as indicated. Then 1.0 ml of 3% albumin–0.75 mm [1-$^{14}$C]palmitate, and ethanol where indicated, was added. An additional 20 min of incubation followed.

[a] Per 20 mg (dry wt)/20 min.

The rate of ethanol utilization by the isolated liver cells was also measured. In two different experiments, liver cells isolated from fasted rats were incubated with an equal volume of 3% albumin–0.75 mm palmitate (see Methods) and ethanol at a final concentration of 100 μg/ml. The rate of ethanol utilization was 74 and 88 nmoles/mg (dry wt)/30 min.

TABLE 5. Effects of ethanol on the conversion of various $^{14}$C-labeled substrates to $^{14}CO_2$ by isolated liver cells

| Substrate | $^{14}CO_2$ Control | $^{14}CO_2$ Ethanol (1 mg/ml) | % Change with Ethanol |
|---|---|---|---|
| | *dpm* | *dpm* | |
| [1-$^{14}$C]Palmitate | 29,980 | 16,870 | −43.7 |
| [1-$^{14}$C]Acetate | 25,550 | 13,950 | −45.4 |
| [6-$^{14}$C]Citrate | 283,400 | 284,670 | +0.4 |
| [1,4-$^{14}$C]Succinate | 301,010 | 255,770 | −15.0 |
| [1-$^{14}$C]Glutamate | 87,260 | 67,550 | −22.6 |
| α-[1-$^{14}$C]Ketoglutarate | 80,770 | 61,280 | −24.1 |

Isolated liver cells from a fasted rat, 26.1 mg (dry wt) in 1.0 ml of suspension medium, were incubated at 37°C for 30 min with 1.0 ml of 3% albumin–0.75 mm [1-$^{14}$C]palmitate (757,100 dpm) with and without ethanol, 1 mg/ml final concentration. $^{14}CO_2$ production was measured and compared with the effect of ethanol on $^{14}CO_2$ production by isolated cells incubated with 1.0 ml of 3% albumin–0.75 mm palmitate and the following substrates at the final concentrations indicated: 100 μm [1-$^{14}$C]acetate (473,680 dpm), 55 μm [6-$^{14}$C]citrate (611,600 dpm), 60 μm [1,4-$^{14}$C]succinate (811,430 dpm), 20 μm [1-$^{14}$C]glutamate (560,500 dpm), and 37 μm [1-$^{14}$C]α-ketoglutarate (557,830 dpm).



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## DISCUSSION

Ethanol clearly enhanced the esterification of palmitate and decreased its oxidation to both ketone bodies and $CO_2$ in the intact liver cells (Table 1 and Fig. 1). On the basis of the known energetics of esterification, $\beta$-oxidation, and the reactions of the citric acid cycle, it has been calculated from the data in Table 1 that the liver cells incubated with ethanol utilized 3 nmoles more ATP/mg (dry wt)/30 min for [1-$^{14}$C]palmitate esterification and derived about 102 nmoles less ATP/mg (dry wt)/30 min from palmitate oxidation than the control cells. The relative energy deficit from [1-$^{14}$C]palmitate metabolism was therefore about 105 nmoles of ATP/mg (dry wt)/30 min.

The ketone body specific activity was about one-half the specific activity of the precursor [1-$^{14}$C]palmitate, indicating that about half of the fatty acids undergoing oxidation in the isolated cells were derived from endogenous lipids. This increases the ATP deficit from FFA metabolism to about 200 nmoles/mg (dry wt)/30 min in the presence of ethanol. Ethanol utilization was about 80 nmoles/mg (dry wt)/30 min (see Results), in close agreement with the value reported by Berry (37). The conversion of this amount of alcohol to acetate could alone yield 480 nmoles of ATP. Although it appears on the basis of other reports (37, 38) that little of the acetate produced from ethanol in the liver undergoes further conversion in this tissue, some conversion of ethanol carbon to ketone bodies is to be expected because it has long been recognized that acetate can be converted, at least in part, to $CO_2$ (39) and ketone bodies (40) in the liver. On the basis of the observed dilution in ketone body specific activity (Table 2), about 22 nmoles of ethanol/mg (dry wt)/30 min was converted to ketone bodies. This represents about 30% of the ethanol utilized. This does not materially affect the energy estimations, which indicate that the ATP obtainable from ethanol oxidation is in excess of the deficit consequent to restricted fatty acid oxidation. Since the oxygen consumption of the isolated cells was unaltered by ethanol (see Results), these energy estimations indicate that alcohol oxidation replaces not only fatty acid oxidation but the oxidation of other substrates as well.

The conversion of palmitate to ketone bodies in the hepatocytes was an order of magnitude greater than its oxidation to $CO_2$ (Table 1). Therefore, the observed 20% decrease in the conversion of palmitate to ketone bodies in the presence of ethanol reflects a decrease of similar magnitude in the $\beta$-oxidation of fatty acids. Ethanol decreased $^{14}CO_2$ production from [1-$^{14}$C]palmitate about 40% (Table 1). It is likely that ketone bodies and $CO_2$ are derived from the same acetyl CoA pool. Based on this assumption, half of the 40% decrease may be ascribed to inhibition of $\beta$-oxidation and half to inhibition of the citric acid cycle. [1-$^{14}$C]Palmitate conversion to $CO_2$ and ketone bodies was, respectively, 3.1 and 30.5 $\mu$g of carbon/20 mg (dry wt)/30 min without ethanol and 1.8 and 23.9 $\mu$g of carbon in the presence of ethanol as estimated from data in Tables 1 and 2. Total ketogenesis was 62.3 and 60 $\mu$g of carbon without and with ethanol, respectively. The total citric acid cycle flux as calculated from these data was 6.3 and 4.5 $\mu$g of carbon/20 mg (dry wt)/30 min without and with ethanol, respectively. Accordingly, the citric acid cycle flux was inhibited 28% by ethanol. This compensates for dilution of the acetyl CoA pool by ethanol and compares well with the 20% inhibition estimated above. The measurement of specific segments of the citric acid cycle (Table 5) indicates inhibition of 23–24% by ethanol.

Pyrazole inhibits liver alcohol dehydrogenase, apparently by forming a dehydrogenase–coenzyme–pyrazole ternary complex (41). It is clear that ethanol metabolism is mandatory for the observed effects of ethanol on esterification and $\beta$-oxidation of palmitate and on the citric acid cycle in the intact liver cells because these effects were completely abolished by preincubating the cells with 1 mM pyrazole (Table 4). Similarly, pyrazole and 4-methylpyrazole almost completely prevented the liver triglyceride accumulation in rats that had been administered ethanol (26, 42, 43).

As the ethanol concentration increased, nearly equal and opposite effects on esterification and total oxidation of palmitate were observed (Fig. 1). The mechanisms by which ethanol elevated esterification and decreased oxidation to $CO_2$ and ketone bodies therefore appear closely related. Ethanol markedly increases the NADH:NAD ratio in the liver cytosol (17, 19, 24). Although energy is immediately available from ethanol oxidation (19), oxygen consumption is unaltered in the presence of long-chain fatty acids (see Results and Ref. 19). Ethanol increased the mitochondrial NADH:NAD ratio in the liver cells as evidenced by the increased $\beta$-hydroxybutyrate:acetoacetate ratio (Table 2). On the basis of these observations and the above calculations, it is suggested that alcohol inhibits the $\beta$-oxidation of fatty acids and the citric acid cycle by competitive oxidation in the intact liver cells and that reducing equivalents produced in the cytosol via the NAD-linked alcohol and aldehyde dehydrogenases are transferred into the mitochondria and thereby inhibit certain NAD-linked oxidations in the mitochondria. The observation of Williamson et al. (19) that oleic acid inhibits the rate of removal of alcohol by the perfused liver, together with the present results, indicates that ethanol oxidation and fatty acid oxidation are mutually competitive; that is, the oxidation of each of these substrates inhibits the oxidation of the other. The oxidation of ethanol by isolated liver



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mitochondria has been reported (44). If a significant amount of ethanol or acetaldehyde is oxidized in hepatocytes by mitochondrial dehydrogenase(s), such a process could contribute to the elevation of the mitochondrial oxidation–reduction potential and to the inhibition of β-oxidation and the citric acid cycle.

The inhibition of β-oxidation by ethanol oxidation is thus consonant with control of fatty acid oxidation by the mitochondrial oxidation–reduction potential. The increased NADH:NAD ratio in the mitochondria consequent to ethanol oxidation may restrict β-oxidation at the acyl CoA dehydrogenase and β-hydroxyacyl CoA dehydrogenase sites. Inhibition at these sites should elevate the long-chain acyl CoA level in the mitochondrial matrix and, in consequence, decrease the vectorial translocation (45, 46) of acyl groups across the inner mitochondrial membrane, resulting in greater availability of acyl CoA in the cytosol. It is suggested that the major cause of the ethanol-induced increase in esterification is enhanced fatty acid availability consequent to restricted fatty acid oxidation. An increased concentration of FFA leads to a more marked increase in triglyceride synthesis than in phospholipid formation (28). Ethanol also mainly increased the conversion of [1-$^{14}$C]palmitate to triglycerides (Table 3 and Fig. 2), supporting the concept that enhanced triglyceride formation resulted from increased fatty acid availability. Ethanol increased esterification of fatty acids to form glycerolipids but did not affect cholesterol esterification (Table 3). The elevation of the hepatic α-glycerophosphate concentration, known to result from ethanol administration (19, 24, 47, 48), may therefore play a role in directing the increased quantity of fatty acids available for esterification into the pathway of glycerolipid formation.

Insight into the mechanism by which ethanol inhibits the citric acid cycle in intact hepatocytes is provided by the effects of ethanol on the oxidation of labeled intermediates (Table 5). The major interaction was observed at the site of α-ketoglutarate oxidation. In view of the report of Garland (49) that pig heart α-ketoglutarate dehydrogenase was inhibited by NADH, the observed ethanol-induced increase in the mitochrondrial NADH:NAD ratio in the intact cells (Table 2), and the inhibition of α-ketoglutarate oxidation in liver cells by β-hydroxybutyrate (50), it is suggested that ethanol oxidation decreased α-ketoglutarate oxidation in the intact hepatocytes by inhibiting α-ketoglutarate dehydrogenase via elevation of the NADH:NAD ratio. Contributing effects of succinyl CoA and guanine nucleotides on α-ketoglutarate dehydrogenase remain possible (51, 52). It is also possible that NADH-stimulated reductive amination of α-ketoglutarate catalyzed by mitochondrial glutamic dehydrogenase is a contributory inhibitory effect of ethanol oxidation on the oxidative decarboxylation of α-ketoglutarate. The decrease in the conversion of [1,4-$^{14}$C]succinate to $^{14}CO_2$, together with the lack of effect of ethanol on the decarboxylation of [6-$^{14}$C]citrate (Table 5), indicates another (albeit minor) site of interaction of ethanol oxidation between succinate and citrate. The major and minor interactions of ethanol oxidation appear to contribute about 80% and 20%, respectively, to the total inhibitory effect on the citric acid cycle activity. Palmitic acid, which increases the mitochondrial NADH:NAD ratio in liver cells (28), also inhibits the citric acid cycle primarily at the site of α-ketoglutarate oxidation (50). Thus, reducing equivalents rapidly generated by substrate oxidations primarily in either the cytosol or the mitochondria of liver cells appear to control the rate of citric acid cycle reactions at this site.

The concentration of ethanol that produced maximum effects on fatty acid metabolism was 50 μg/ml (Fig. 1). Rats fed a diet containing 5% ethanol have blood alcohol concentrations of about 600 μg/ml (53). The level of ethanol in the blood of human subjects after the oral administration of only 0.2 g of ethanol/kg body weight is about 250 μg/ml (54, 55). It is therefore likely that these levels of alcohol intake exceed the dose that produces maximum effects on the oxidation and esterification of long-chain fatty acids and on the citric acid cycle activity in the liver.

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