Metabolic Effects of Ethanol in Perfused Rat Liver*

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JOHN R. WILLIAMSON,† ROLAND SCHOLZ, EDWARD T. BROWNING,§ RONALD G. THURMAN,¶ AND MIRIAM H. FUKAMI

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

SUMMARY

In the present study, ethanol oxidation by the perfused rat liver has been used to investigate the interrelationships between the pathways of glucose metabolism, fatty acid oxidation, and the citric acid cycle. In the absence of exogenous fatty acids, the production of glucose from alanine was stimulated 2-fold by 10 mm ethanol, whereas, in the presence of 1 mm oleate, ethanol caused an inhibition of net glucose production. Measurements of the rates of ethanol utilization and acetate formation showed that over 80% of the ethanol metabolized was converted to acetate. The increased rate of generation of reducing equivalents in the cytosol during ethanol oxidation increased the oxidation-reduction state of pyridine nucleotides in both the intramitochondrial and extramitochondrial compartments. This fact was established by analyses of the tissue content of pyridine nucleotides and substrate couple ratios, and directly by surface fluorometry. Changes of flavin and pyridine nucleotide fluorescence intensity from the surface of the liver showed that the transfer of reducing equivalents from cytosol to mitochondria during ethanol oxidation was very rapid.

Analyses of intermediates in the gluconeogenic pathway of livers perfused in the absence of fatty acids indicated an activation site at the glyceraldehyde-3-P dehydrogenase step upon ethanol addition. The stimulatory effect of ethanol on gluconeogenesis from alanine, therefore, results from the increased availability of the NADH in the cytosol. On the other hand, when ethanol was added to livers perfused in the presence of oleate, an inhibitory site was observed between fructose-1,6-di-P and fructose-6-P. Measurement of the tissue levels of the known modifiers of phosphofructokinase and fructose diphosphate phosphatase indicated that this effect was caused primarily by deactivation of phosphofructokinase resulting from a fall of the citrate content.

Oxidation of NADH produced during ethanol metabolism inhibited the activity of the citric acid cycle. Sites of inhibition were identified at the citrate synthase and isocitrate dehydrogenase steps. The relative strengths of the inhibitory interactions at these sites were dependent on the rate of $\beta$ oxidation. It is proposed that a coordinated inhibition of citrate synthase and isocitrate dehydrogenase is mediated primarily by the increased state of reduction of intramitochondrial pyridine nucleotides.

In previous studies from this laboratory, in which alanine was used as a gluconeogenic precursor (1, 2), it was found that oleate stimulated the formation of glucose and ketone bodies by the perfused rat liver. Changes in the contents of intermediates of the gluconeogenic pathway in the presence and absence of oleate indicated an interaction site between 3-P-glycerate and the triose phosphates. Simultaneously, the NADH:NAD + ratio in both cytosolic and mitochondrial compartments increased. These results were interpreted as indicating a stimulation of gluconeogenesis by an increased availability of NADH to glyceraldehyde-3-P dehydrogenase. In contrast, when lactate or pyruvate were used as gluconeogenic precursors (2-4), forward crossovers were obtained between pyruvate and oxaloacetate, indicating stimulation of pyruvate carboxylase, probably because of the increased acetyl coenzyme A content (3 5). The differences in the metabolite patterns upon fatty acid addition in the presence of different substrates can be interpreted on the supposition that the transfer of reducing equivalents from the mitochondria to the cytosol was rate-limiting when alanine was the gluconeogenic precursor, and that this process was stimulated by fatty acid oxidation (2).

Oxidation of ethanol by the liver also increases the supply of reducing equivalents in the cytosol (6), and provides another means of investigating the effect of NADH availability on the rate of gluconeogenesis. Studies with liver slices from fed (but not fasted) rats incubated with 10 mm alanine have shown that gluconeogenesis was stimulated by ethanol (7). Although the rates of glucose production are much lower with liver slices than with perfused liver (8), the potential importance of the cytosolic NAD oxidation-reduction potential in the control of gluconeogenesis was illustrated by this work.

The administration of ethanol to humans and dogs was found to have inconsistent effects on the blood glucose levels unless the subjects were fasted for several days, in which case hypo glycemia developed (9, 10). Other data from experiments in vivo suggested that the inhibitory effect of ethanol on glucose
production was dependent on the concomitant oxidation of fatty acids, whereas the stimulatory effect was only observed when fatty acid oxidation was low, and substrate availability high. Furthermore, a positive effect of ethanol in gluconeogenesis is to be expected only with substrates more oxidized than glucose, i.e., pyruvate, or those producing reducing equivalents in mitochondria rather than in the cytosol during their metabolism, i.e., alanine (11).

In the present experiments, the effects of ethanol were investigated in livers perfused with alanine as substrate, either in the absence or presence of 1 mM oleate. Metabolites were measured in the perfusion fluid and in the liver tissue in order to determine the nature of the interactions between ethanol oxidation, fatty acid oxidation, citric acid cycle activity, and gluconeogenesis. A preliminary account of part of this work has been published (12).

**EXPERIMENTAL PROCEDURES**

**Animals**—Male albino rats of Holtzman strain, 180 to 200 g in weight, were used. Food was withheld from the animals 24 to 30 hours prior to the perfusion experiments.

**Liver Perfusion**—Perfusion techniques were the same as described previously (1, 2, 5, 13). The perfusion fluid was Krebs-Henseleit bicarbonate buffer containing 4 g/100 ml of bovine serum albumin (Fraction V, Sigma). Fatty acids were removed from the albumin by charcoal treatment (14). The perfusion fluid was dialyzed against Krebs-Henseleit bicarbonate buffer, and filtered through Millipore filters (0.45 µm porosity) prior to use. The pH of the fluid after equilibration with 95% O2-5% CO2 in the oxygenator was 7.4. Alanine was added to give an initial concentration of 5 mM, and was infused continuously at a rate of about 17 µmoles per min, which maintained the alanine concentration between 5 and 10 mM throughout the experiment. Although this alanine concentration was unphysiologically high, it was needed in the present experiments in order to prevent the liver from becoming substrate depleted. Oleic acid (chromatographically pure from Applied Science Laboratory, Inc., State College, Pennsylvania) was infused as an emulsion complexed with albumin (1). Ethanol was added over a 3-min interval to give an initial concentration of 10 mM.

**Analytical**—Samples of perfusion fluid were removed every 15 min, deproteinized with perchloric acid, and analyzed for glucose, lactate, pyruvate, acetoacetate, β-hydroxybutyrate, urea, and ethanol by standard enzyme procedures (15). Fatty acids and acetate were determined by gas chromatography (16). At the end of 90 min of perfusion, the livers were rapidly frozen with large aluminum tongs cooled in liquid Nz. Portions of the tissue were powdered and extracted with alcoholic perchloric acid or alcoholic KOH as described by Williamson and Corkey (17). The perichondrial acid extracts, after neutralization with 6 N K2CO3, were used for the measurement of the glycolytic intermediates, adenine nucleotides, NAD+ and NADP+, CoA and acetyl-CoA, and the intermediates of the citric acid cycle. Alkaline extracts were used for the measurement of NADH and NADPH. Long chain fatty acyl-CoA was measured as CoA after alkaline hydrolysis of the perichondrial acid-insoluble material (17). All intermediates were assayed using fluorometric enzyme procedures which are fully described elsewhere (17, 18). Oxygen concentrations in the effluent perfusion fluid were continuously measured using a platinum electrode (19).

**Surface Fluorometry**—The use of a double fluorometer for the simultaneous measurement of pyridine nucleotide and flavin oxidation-reduction changes from the surface of the hemoglobin-free perfused rat liver is described elsewhere (19).

**RESULTS**

**Metabolic Changes in Perfusion Fluid**—The effects of ethanol on glucose production from alanine by perfused rat liver. The volume of perfusion fluid was initially 100 ml, and 2-ml samples were removed at 15-min intervals. The perfusion fluid contained alanine at an initial concentration of 5 mM. Alanine was also infused continuously at a rate of 16.7 µmoles per min throughout the experiment. Oleate was added after 30 min of perfusion. The rate of oleate addition was 33 µmoles per min for 3 min, followed by 3 µmoles per min until perfusion was terminated. Ethanol was added after 60 min of perfusion to give an initial concentration of 10 mM. Vertical bars, 2 S.E.M., with six or eight livers in each group. In these experiments, 100 g, body weight, were equivalent to 3.5 g, wet weight, of liver.
Fig. 2. Effects of ethanol in the presence and absence of oleate on lactate production from alanine by perfused rat liver. Oleate was added after 30 min, and ethanol after 60 min of perfusion. Vertical bars, 2 S.E.M., with six or eight livers in each group.

When ethanol was added in the presence of oleate, the ratio of 3-hydroxybutyrate to acetoacetate increased gradually over the 30-min interval to 1.8.

The concentration of free fatty acids in the perfusion fluid containing charcoal-treated albumin was 0.02 μM. Oleate infusion maintained the concentration at about 1.0 μM (Fig. 5). Subsequent administration of ethanol had little effect on the perfusate free fatty acid concentration.

Rates of ethanol utilization and acetate production by perfused rat livers are shown in Fig. 6. Oleate diminished ethanol consumption by about 25%. Acetate formation accounted for 88% and 90% of the ethanol uptake in the absence and presence of oleate, respectively. Only trace amounts of acetaldehyde could be detected in the perfusion fluid. These results show that ethanol is oxidized almost quantitatively to acetate by the perfused rat liver.

Oxygen Consumption—Rates of oxygen consumption by perfused livers are shown in Fig. 7. The oxygen concentration in the perfusion fluid leaving the liver was measured polarographically. The rates of oxygen utilization were calculated from the arteriovenous differences and the flow rate. Addition of alanine increased the rate of oxygen consumption from 750 to 1100 μatoms of oxygen per 100 g, body weight, per hour, whereas ethanol increased it further to 1300 μatoms of oxygen per 100 g, body weight, per hour (Curve A). Oleate added after alanine...

(Curves B and C) increased the rate of oxygen consumption from 1100 to 1600 μatoms of oxygen per 100 g, body weight, per hour, but subsequent addition of ethanol caused a gradual decrease (Curve B).

Intermediates of Gluconeogenesis—In the absence of exogenous fatty acids, ethanol produced a significant decrease in the tissue contents of intermediates in the sequence from lactate to 3-P-glycerate, and a significant increase of intermediates in the sequence from glyceraldehyde-3-P to glucose-6-P (Table 1). These changes, together with those of malate and oxaloacetate, are presented in the form of a crossover plot in Fig. 8. The tissue contents after ethanol addition are expressed as a percentage of the values observed in control livers (see Reference 2). Malate levels increased in accordance with a shift of the NADH:NAD+ ratio toward a more reduced state. Since flux in the direction of glucose synthesis increased, the forward crossover between 3-P-glycerate and glyceraldehyde-3-P indicates an activational site at the glyceraldehyde-3-P dehydrogenase step. This crossover is presumably caused by an interaction at the bimolecular dehydrogenase reaction caused by the elevated NAD+ concentration in the cytosol (2).

After addition of ethanol to perfused livers in the presence of oleate, the tissue content of lactate remained unchanged, whereas the contents of pyruvate, P-enolpyruvate, 2-P-glycerate, 3-P-

![Fig. 6. Utilization of ethanol (left) and production of acetate (right) by rat livers perfused with alanine in the presence and absence of oleate. The perfusion conditions were similar to those of previous figure. Oleate was added after 30 min and ethanol after 60 min of perfusion. Vertical bars, 2 S.E.M., with six livers in each group.

![Fig. 7. Rate of oxygen consumption by the isolated perfused rat liver. Additions to 100 ml of perfusion fluid were made as denoted on the figure. Curve A, rate of oxygen consumption following addition of alanine after 15 min and ethanol after 75 min of perfusion. Curve B, rate of oxygen consumption following addition of alanine after 15 min, oleate after 45 min, and ethanol after 75 min of perfusion. Curve C, rate of oxygen consumption following addition of alanine after 15 min, and oleate after 45 min of perfusion. Shaded area, 2 S.E.M.](http://www.jbc.org/doi/10.1042/mbib0047)

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Control</th>
<th>Ethanol</th>
<th>Difference</th>
<th>Oleate</th>
<th>Oleate plus ethanol</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>4750 ± 256</td>
<td>3880 ± 200</td>
<td>-870 ± 0</td>
<td>2030 ± 218</td>
<td>2040 ± 151</td>
<td>10</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>876 ± 50</td>
<td>254 ± 33</td>
<td>-622 ± 0</td>
<td>174 ± 8</td>
<td>100 ± 8</td>
<td>-10</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>816 ± 92</td>
<td>392 ± 22</td>
<td>-424 ± 0</td>
<td>724 ± 35</td>
<td>218 ± 13</td>
<td>-511</td>
</tr>
<tr>
<td>2-P-glycerate</td>
<td>148 ± 14</td>
<td>86 ± 6</td>
<td>-65 ± 0</td>
<td>139 ± 7</td>
<td>56 ± 4</td>
<td>-88</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>1593 ± 193</td>
<td>717 ± 46</td>
<td>-876 ± 0</td>
<td>1356 ± 90</td>
<td>373 ± 31</td>
<td>-988</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P</td>
<td>6.8 ± 1.0</td>
<td>13.5 ± 1.3</td>
<td>6.7 ± 0</td>
<td>8.6 ± 1.0</td>
<td>11.6 ± 1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>67.2 ± 4.1</td>
<td>85.3 ± 3.4</td>
<td>18.1 ± 0</td>
<td>72.3 ± 1.7</td>
<td>83.6 ± 2.7</td>
<td>11.3</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>266 ± 37</td>
<td>652 ± 56</td>
<td>386 ± 0</td>
<td>500 ± 42</td>
<td>1150 ± 110</td>
<td>650</td>
</tr>
<tr>
<td>Fructose-1,6-di-P</td>
<td>12.9 ± 1.1</td>
<td>21.5 ± 1.6</td>
<td>8.6 ± 0</td>
<td>19.0 ± 1.1</td>
<td>26.5 ± 3.1</td>
<td>10.5</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>40 ± 1.7</td>
<td>58 ± 3.7</td>
<td>18 ± 0</td>
<td>59 ± 3.4</td>
<td>45 ± 2.8</td>
<td>-14</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>104 ± 8</td>
<td>170 ± 14</td>
<td>71 ± 0</td>
<td>202 ± 16</td>
<td>158 ± 14</td>
<td>-44</td>
</tr>
</tbody>
</table>

* By t test for significance, p < 0.01.

Table 1. Effects of ethanol in presence and absence of oleate on intermediates of gluconeogenic pathway

Livers from fasted rats were perfused with Krebs-Henseleit bicarbonate buffer containing 4% albumin and, initially, 5 mM alanine. Alanine was also infused continuously at the rate of 16.7 μmoles per min for 90 min. When present, oleate was added at a rate of 33 μmoles per min for 3 min after 30 min of perfusion, followed by 2.7 μmoles per min for 60 min. Ethanol was added after 60 min of perfusion to give a final concentration of 10 mM. After 90 min of perfusion, the livers were rapidly frozen with tongs cooled in liquid N₂. Values shown are the mean ± standard error of the mean of 8 or 10 livers.
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FIG. 8. Effects of ethanol on intermediates of the gluconeogenic sequence in rat livers perfused with alanine in the absence of exogenous fatty acids. The tissue contents of the intermediates are given in Table I. Vertical bars, 2 S.E.M., with six livers in each group. Lact, lactate; Mal, malate; PEP, P-enolpyruvate; 3PGA, 3-P-glycerate; DAP, dihydroxyacetone-P; F6P, fructose-6-P; Pyr, pyruvate; OAA, oxalacetate; GAP, glyceraldehyde-3-P; G6P, glucose-6-P; GAPDH, glyceraldehyde-3-P dehydrogenase.

FIG. 9. Effects of ethanol on intermediates of the gluconeogenic sequence in rat livers perfused with alanine in the presence of 1 mM oleate. The tissue contents of the intermediates are given in Table I. Vertical bars, 2 S.E.M., with eight livers in each group. PFK, phosphofructokinase; FDPase, fructose-1,6-di-P phosphatase. For other abbreviations, see legend to Fig. 8.

glycerate, fructose-6-P, and glucose-6-P decreased, and the contents of the triose phosphates and fructose-1,6-di-P increased (Table I). Since the net flux decreased, a forward crossover is identified between fructose-1,6-di-P and fructose-6-P (Fig. 9), indicating an inhibitory site for net glucose production, which could represent either an activation of phosphofructokinase or an inhibition of fructose diphosphate phosphatase.

Pyridine Nucleotide Oxidation-Reduction Changes—Analysis of the tissue contents of pyridine nucleotides showed that ethanol, oleate, and oleate plus ethanol increased the NADH level by 250, 180, and 290%, respectively, as compared with control livers (Table II). The levels of NADPH also increased after ethanol or oleate administration. However, this change is best seen from the statistically significant fall of NADP+.

The enhanced supply of reducing equivalents produced by either ethanol or fatty acid oxidation increased the ratios of lactate to pyruvate, α-glycerophosphate to dihydroxyacetone-P, malate to oxalacetate, and β-hydroxybutyrate to acetoacetate (Table III). Ethanol added alone was about as effective as oleate, but oleate plus ethanol produced the largest changes of the substrate couple ratios, in accordance with changes of total pyridine nucleotides. Since lactate dehydrogenase is entirely cytosolic in its distribution, whereas β-hydroxybutyrate dehydrogenase is located in the mitochondria, the observed increases in the ratios of lactate to pyruvate and β-hydroxybutyrate to acetoacetate indicate that ethanol and fatty acid oxidation result in a higher state of reduction of both the cytosolic and mitochondrial NAD systems. Even though the calculated oxidation-reduction potential of the mitochondrial NAD system is about 40 mV more negative than that of the cytosol, the data show that the flow of reducing equivalents is bidirectional, since the production of NADH during ethanol oxidation occurs in the cytosol, whereas that during fatty acid oxidation occurs in the mitochondria.

The kinetic data of the oxidation-reduction changes produced by ethanol in the presence and absence of oleate were investigated further by the simultaneous measurement of surface fluorescence changes attributable to flavins and pyridine nucleotides (19). An upward deflection in Figs. 10 and 11 represents the reduction of flavoproteins (lower traces) and pyridine nucleotides (upper traces). The pyridine nucleotide fluorescence changes are derived from both cytosolic and mitochondrial spaces, whereas the flavin fluorescence changes are predominantly due to mitochondrial flavoproteins (19). At the left-hand side of Fig. 10, a brief cycle of anoxia is shown for calibration purposes. Addition of 10 mM alanine to the perfusion fluid had little effect on the oxida-
TABLE III
Effects of ethanol in presence and absence of oleate on oxidation-reduction state of pyridine nucleotides in perfused rat liver

The perfusion conditions were the same as described in Table I. The oxidation-reduction potential of the NAD system is calculated using values of -337, -215, -197, and -297 mv for the midpotentials of the NADH:NAD+, lactate-pyruvate, malate-oxalacetate, and β-hydroxybutyrate-acetoacetate oxidation-reduction systems, respectively, at 38° and an ionic strength of 0.25 M (20). Values shown are the means ± standard error of the mean with 8 to 10 livers in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate oxidation-reduction couples</th>
<th>NAD⁺ oxidation-reduction potential calculated from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio of lactate to pyruvate</td>
<td>Ratio of α-glycero-phosphate to dihydroyzocine-P</td>
</tr>
<tr>
<td>Control</td>
<td>5.3 ± 0.3</td>
<td>4.38 ± 0.70</td>
</tr>
<tr>
<td>Ethanol</td>
<td>12.9 ± 1.1</td>
<td>7.72 ± 0.68</td>
</tr>
<tr>
<td>Difference</td>
<td>7.6</td>
<td>3.34</td>
</tr>
<tr>
<td>Oleate</td>
<td>11.8 ± 1.6</td>
<td>7.10 ± 0.53</td>
</tr>
<tr>
<td>Oleate plus ethanol</td>
<td>20.3 ± 1.5</td>
<td>13.7 ± 1.1</td>
</tr>
<tr>
<td>Difference</td>
<td>8.5</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Fig. 10. Changes of pyridine nucleotide and flavin reduction as measured by surface fluorometry in the perfused rat liver. Reduction of pyridine nucleotide (fluorescence increase) is measured by an upward deflection of the upper trace. Reduction of flavin (fluorescence decrease) is measured by an upward deflection of the lower trace. Pyridine nucleotide fluorescence was measured using primary and secondary filters with maximum transmission at wave lengths of 366 and 450 nm, respectively. Flavin fluorescence was measured with primary and secondary filters with transmission maxima at 436 and 570 nm, respectively. Changes of fluorescence refer to the initial aerobic levels, which are arbitrarily taken as 100%. The times of addition of alanine and ethanol are noted in the figure.

Fig. 11. Effects of oleate and ethanol on pyridine nucleotide and flavin fluorescence from the surface of rat liver perfused with alanine as substrate. See legend to Fig. 9 for further details. Oleate and ethanol were added after 60 and 72 min, as indicated in the figure.
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Effects of Ethanol in Presence and Absence of Oleate on Levels of Ketone Bodies in Perfused Rat Livers

The perfusion conditions were the same as described in Table I. Values shown are the means ± standard error of the mean with 8 to 10 livers in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>β-Hydroxybutyrate</th>
<th>Acetoacetate</th>
<th>Total Ketone Bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.66 ± 0.10</td>
<td>2.04 ± 0.17</td>
<td>2.70 ± 0.19</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.15 ± 0.13</td>
<td>1.60 ± 0.10</td>
<td>2.75 ± 0.10</td>
</tr>
<tr>
<td>Difference</td>
<td>0.49*</td>
<td>-0.44*</td>
<td>0.05</td>
</tr>
<tr>
<td>Oleate</td>
<td>5.89 ± 0.95</td>
<td>7.92 ± 1.97</td>
<td>13.81 ± 2.07</td>
</tr>
<tr>
<td>Oleate plus ethanol</td>
<td>14.63 ± 2.26</td>
<td>7.21 ± 1.04</td>
<td>21.84 ± 2.52</td>
</tr>
<tr>
<td>Difference</td>
<td>8.74*</td>
<td>-0.71</td>
<td>8.03*</td>
</tr>
</tbody>
</table>

* By t test for significance, p < 0.01.

Effects of Ethanol on Intermediates of Citric Acid Cycle in Rat Livers Perfused in Presence and Absence of Oleate

The perfusion conditions were the same as described in Table I. Values shown are the means ± standard error of the mean with 4 livers.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Malate</th>
<th>Oxalacetate</th>
<th>Citrate</th>
<th>α-Ketoglutarate</th>
<th>Succinate</th>
<th>Glutamate</th>
<th>Aspartate</th>
<th>Aspartate X α-Ketoglutarate/ Glutamate X Oxalacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>361 ± 23</td>
<td>12.7 ± 1.0</td>
<td>819 ± 56</td>
<td>1118 ± 85</td>
<td>891 ± 129</td>
<td>27.64 ± 1.39</td>
<td>4.10 ± 0.36</td>
<td>10.6 ± 0.9</td>
</tr>
<tr>
<td>Ethanol</td>
<td>693 ± 46</td>
<td>10.7 ± 0.8</td>
<td>821 ± 39</td>
<td>663 ± 57</td>
<td>423 ± 117</td>
<td>34.04 ± 1.87</td>
<td>10.18 ± 0.65</td>
<td>11.5 ± 1.0</td>
</tr>
<tr>
<td>Difference</td>
<td>332a</td>
<td>-2.0</td>
<td>2</td>
<td>-455b</td>
<td>-408b</td>
<td>6.4a</td>
<td>6.08b</td>
<td>0.9</td>
</tr>
<tr>
<td>Oleate</td>
<td>668 ± 42</td>
<td>16.6 ± 1.0</td>
<td>1800 ± 290</td>
<td>502 ± 55</td>
<td>1600 ± 117</td>
<td>47.05 ± 4.07</td>
<td>13.11 ± 1.54</td>
<td>0.1 ± 0.7</td>
</tr>
<tr>
<td>Oleate plus ethanol</td>
<td>1458 ± 126</td>
<td>13.6 ± 0.5</td>
<td>1117 ± 133</td>
<td>332 ± 42</td>
<td>981 ± 308</td>
<td>32.93 ± 3.34</td>
<td>14.52 ± 1.73</td>
<td>10.4 ± 1.0</td>
</tr>
<tr>
<td>Difference</td>
<td>4900</td>
<td>-3.0</td>
<td>-683p</td>
<td>-260p</td>
<td>-628p</td>
<td>-14.10p</td>
<td>1.41</td>
<td>1.3</td>
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</tbody>
</table>

a Mean of four livers.

b By t test for significance, p < 0.02.
and is presumably oxidized primarily by cytosolic dehydrogenases. Thus, 2 molecules of NADH are generated in the cytosol is shown by the fluorometric observation of mitochondrial flavin increases the state of reduction of the NAD+ dehydrogenase systems. Reducing equivalents produced in the cytosol are trans-

In the present experiments, acetate production accounted for more than 80% of the ethanol consumption. Acetaldehyde, the product of the first oxidation step, does not accumulate (cf. References 25 and 26 and Fig. 6), and is presumably oxidized primarily by cytosolic dehydrogenases. Thus, 2 molecules of NADH are generated in the cytosol per molecule of ethanol oxidized. As demonstrated here, and previously by many other workers (e.g. 6, 7, 13, 27), ethanol increases the state of reduction of the NAD+ dehydrogenase systems. Reducing equivalents produced in the cytosol are transported into the mitochondria where they are oxidized in the respiratory chain. The existence of a rapid transport mechanism of malate as the hydrogen carrier, coupled with transamination between oxalacetate and glutamate to form α-ketoglutarate and aspartate, which then traverse the membrane and undergo reverse transamination to provide oxalacetate as an extramitochondrial hydrogen acceptor. Thus, net NADH entry into the mitochondria is thought to be associated with an ingress of malate and glutamate and an egress of α-ketoglutarate and aspartate (21, 31). The transamination steps are a basic requirement for the regeneration of extramitochondrial oxalacetate; first, because of the relative impermeability of the mitochondrial membrane to oxalacetate, and second, because of the adverse oxalacetate concentration gradient (4, 21). Although there is no conclusive proof that this shuttle mechanism is operative in the intact liver, a number of experiments provide indirect support for it as opposed to the α-glycerophosphate shuttle (21, 32).

**Effects of Ethanol on Gluconeogenesis—**Data presented in this paper show that gluconeogenesis from alanine was increased by ethanol in the absence of exogenous fatty acids. Analyses of intermediates in the gluconeogenic pathway identified a forward crossover between 3-P-glycerate and glyceraldehyde-3-P, indicating a site of activation at the glyceraldehyde-3-P dehydrogenase step. A similar site of interaction was observed after addition of oleate to livers perfused with alanine (1), and is confirmed by the present results (Table I). Oleate and ethanol both serve as readily available sources for the generation of reducing equivalents, even though the subcellular location of the primary dehydrogenase step is different. Net glucose formation is increased by about the same amount with either treatment, but whereas oleate significantly increased acetyl-CoA levels, ethanol did not (Table VI). Thus, possible activation of pyruvate carboxylase by acetyl-CoA cannot explain the increased rate of glucose production (2, 38). This can only be brought about by the elevated cytosolic NADH concentration interacting with glyceraldehyde dehydrogenase.

When ethanol was added to livers after prior addition of oleate, the rate of glucose formation decreased. The metabolite changes shown in Fig. 9 suggested that this effect was not caused by restriction of the pyruvate carboxylase reaction as a result of the lowered pyruvate levels, as proposed by other workers (34), but could be caused either by inhibition of fructose diphosphate phosphatase or stimulation of phosphofructokinase. In vitro, fructose diphosphate phosphatase is inhibited by AMP (35, 36), whereas phosphofructokinase is activated by ADP and AMP, and inhibited by ATP and citrate (37, 38). However, an exam-

**DISCUSSION**

**Intracellular Hydrogen Transport during Ethanol Oxidation—**In liver, ethanol is metabolized almost quantitatively to acetate both in vivo and in vitro (22-25). In the present experiments, acetate production accounted for more than 80% of the ethanol consumption. Acetaldehyde, the product of the first oxidation step, does not accumulate (cf. References 25 and 26 and Fig. 6), and is presumably oxidized primarily by cytosolic dehydrogenases. Thus, 2 molecules of NADH are generated in the cytosol per molecule of ethanol oxidized. As demonstrated here, and previously by many other workers (e.g. 6, 7, 13, 27), ethanol increases the state of reduction of the NAD+ dehydrogenase systems. Reducing equivalents produced in the cytosol are transported into the mitochondria where they are oxidized in the respiratory chain. The existence of a rapid transport mechanism is shown by the fluorometric observation of mitochondrial flavin reduction, which occurred almost immediately after ethanol entered the liver (Figs. 10 and 11).

As first demonstrated by Lehninger with isolated liver mitochondria (28, 29), NADH does not readily pass through the mitochondrial membrane. It has been proposed that reducing equivalents are transferred through this barrier by various substrate hydrogen shuttles (30). One possibility involves the transport of malate as the hydrogen carrier, coupled with transamination between oxalacetate and glutamate to form α-ketoglutarate and aspartate, which then traverse the membrane and undergo reverse transamination to provide oxalacetate as an extramitochondrial hydrogen acceptor. Thus, net NADH entry into the mitochondria is thought to be associated with an ingress of malate and glutamate and an egress of α-ketoglutarate and aspartate (21, 31). The transamination steps are a basic requirement for the regeneration of extramitochondrial oxalacetate; first, because of the relative impermeability of the mitochondrial membrane to oxalacetate, and second, because of the adverse oxalacetate concentration gradient (4, 21). Although there is no conclusive proof that this shuttle mechanism is operative in the intact liver, a number of experiments provide indirect support for it as opposed to the α-glycerophosphate shuttle (21, 32).
Preciable increase of ammonia in the perfusion fluid could be de-
molecule of glucose synthesized by gluconeogenesis. No ap-
rect for the amount of lactate and glucose formed. These ex-
periments, urea formation was used as a measure of alanine
crease in the amount of energy consumed for glucose formation
between fructose-1,6-P and fructose-6-P. This would entail an
observed after addition of ethanol in the presence of oleate, how-
possible allosteric interactions with fructose diphosphate phos-
between the directional changes of the adenine nucleotides and
calculations assume that 1 molecule of urea is formed for every
Table VIII. The net metabolic balances of glucose, lactate, /3-
to calculate approximate flux rates of the citric acid cycle un-
the over-all metabolic balance of the perfused livers, it is possible
tion of Tables I and VII shows that there is no correlation
between the directional changes of the adenine nucleotides and
possible allosteric interactions with fructose diphosphate phos-
phosphate or phosphofructokinase. The decreased citrate content
observed after addition of ethanol in the presence of oleate, how-
ever, could cause an increased activity of phosphofructokinase.
On this basis, the decreased rate of net glucose production would be
accounted for mainly by an increased rate of recycling be-
fructose-1,6-P and fructose-6-P. This would entail an
increase in the amount of energy consumed for glucose formation
(8). The changes in oxygen consumption after ethanol addition to
the liver in the presence of oleate are consistent with the exist-
ence of a futile energy-consuming cycle, since the rapid decrease of
glucose production (Fig. 1) is associated with a delayed and
relatively small decrease of oxygen consumption (Fig. 7).
Calculation of Citric Acid Cycle Flux—From a knowledge of
the over-all metabolic balance of the perfused livers, it is possible
to calculate approximate flux rates of the citric acid cycle under
different metabolic conditions. These calculations are shown in
Table VIII. The net metabolic balances of glucose, lactate, /3-
hydroxybutyrate, acetoacetate, and urea across the liver over the
time intervals shown are given in Lines 1 to 5. In these ex-
periments, urea formation was used as a measure of alanine
utilization. Line 6 shows the excess urea formation after cor-
rection for the amount of lactate and glucose formed. These
calculations assume that 1 molecule of urea is formed for every
molecule of glucose synthesized by gluconeogenesis. No ap-
niciable increase of ammonia in the perfusion fluid could be de-
tected. The rate of oxygen consumption by the liver is shown in
Line 7. This is corrected in Line 8 for the cyanide-insensitive respiration, which is estimated to be 13% of the basal oxygen
uptake in the absence of exogenous substrate (39). Lines 9 and
10 give the oxygen equivalents for the measured formation of
acetoacetate and /3-hydroxybutyrate, assuming an average fatty
acid chain length of C16, while the total respiration associated
with ketone production is shown in Line 11. Line 12 shows the
oxygen equivalents required for the oxidation of the 2 moles of
NADH which are produced per mole of ethanol oxidized. The
alanine equivalent of the excess urea is assumed to be oxidized in
the citric acid cycle. A small correction is needed for the oxygen equivalents consumed in the conversion of alanine to acetyl-CoA
as shown in Line 13. The oxygen equivalents for the complete
oxidation of fatty acids to CO2 (Line 14) is calculated by sub-
tracting the sum of Lines 11, 12, and five times Line 13 from the
cyanide-sensitive respiration. The proportion of this respiration
associated with B oxidation (90% of the total fatty acid respira-
tion) is shown in Line 15. The total cycle-independent respiration
is calculated from the sum of Lines 11, 12, 13, and 15 and is
given in Line 16. The cycle-dependent respiration (Line 17) is
obtained by subtracting the cycle-independent respiration from
the cyanide-sensitive respiration. Cycle flux (Line 18) is ex-
pressed as one-fourth of cycle-dependent respiration. The cal-
culated rate of 175 pmoles per 100 g, body weight, per hour, with
alanine as substrate is about 30% greater than that previously
calculated for livers perfused with lactate (6). As previously
found (5), oleate slightly increased the flux of the citric acid cycle.
Effects of Ethanol on Citric Acid Cycle—Calculations given in
Table VIII show that ethanol oxidation inhibited citric acid cycle
activity by about 75%, both in the presence and absence of
exogenous fatty acids. This effect is in accordance with the de-
creased CO2 production and respiratory quotient observed in
livers after addition of ethanol (6, 40). The mitochondrial oxida-
tion of NADH produced from ethanol influences both the
oxidation-reduction state and the energy state of the liver. These
secondary changes are probably responsible for the ob-
served interactions in the citric cycle. Changes in the citrate
content of the liver in turn affect the rate of glucose production,
as discussed above.

In the absence of exogenous fatty acids, ethanol oxidation de-
creased the contents of a-ketoglutarate and succinate, but did not
change the citrate content. If the fall of total tissue a-
ketoglutarate is reflected by a fall in the mitochondrial space, an
inhibition of isocitrate dehydrogenase is indicated. Since the
formation of citrate by citrate synthase is essentially irreversible
under physiological conditions, an inhibition only at isocitrate
dehydrogenase would cause an accumulation of citrate. The
fact that this does not happen indicates that citrate synthase
must also be inhibited. Such a coordinated control at the first
two steps of the citric acid cycle produces a stronger interaction,
and also permits added flexibility in the control of the citrate
level. In the presence of oleate, on the other hand, ethanol caused a
fall of the citrate content in addition to decreased contents of
a-ketoglutarate and succinate. These changes indicate primary
inhibition at the citrate synthase site. However, interpretation
of results of analyses of the total tissue content of intermediates
is difficult since citric acid cycle intermediates are present in both
cytosolic and mitochondrial spaces. If these metabolites were
distributed uniformly, the content in the cytosol would be about
10 times that in the mitochondria. Consequently, deductions from total tissue measurements about changes of metabolites in the mitochondrial space can be misleading. Thus, although it is suggested here that the fall of the α-ketoglutarate content during ethanol oxidation is reflected by a decreased concentration in the mitochondrial space, it is probable that the decrease in α-ketoglutarate content observed after oleate addition occurs mainly in the cytosol (5). This difference is inferred from the fact that oleate, unlike ethanol, does not cause a decrease of cycle flux and, therefore, would not be expected to produce large decreases of the mitochondrial α-ketoglutarate concentration.

Studies with purified citrate synthase and isocitrate dehydrogenase have revealed a number of control properties which could account for a coordination of control at these enzyme sites. One possibility, which has been advanced by Atkinson (41), Jangaard, Unkeless, and Atkinson (42), Shepherd, Yates, and Garland (43-45) is based on control by the intramitochondrial ATP:ADP ratio, or more specifically, by the adenine nucleotide energy charge (46). However, recent studies with isolated rat liver mitochondria (47-51) have failed to substantiate this proposal. A second possibility for control of citrate synthase activity is inhibition by long chain fatty acyl-CoA derivatives (52, 53). However, tissue analyses of fatty acyl-CoA given in Table VI show them to decrease rather than increase after ethanol addition. These observations, and the arguments presented by other workers (45, 54), do not favor the role of long chain acyl-CoA as an essential modulator of citrate synthase in the intact liver cell.

Finally, a third possibility involves control by changes of the pyridine nucleotide oxidation-reduction potential, which may be concluded from the observations that the NAD-linked isocitrate dehydrogenase is strongly inhibited by NADH, and that this inhibition is potentiated by NADPH (55, 56). The NADP+-linked enzyme is probably controlled by NADP+ availability (45), because of the slow rate of transhydrogenation from NADPH to NAD+ in liver (57).

In the present experiments, addition of ethanol to livers perfused in the absence of exogenous fatty acids produced an appreciable change of the ATP:ADP ratio, as calculated from total tissue analyses, but markedly increased the ratios of NADH: NAD+ and NADPH:NADP+. It is suggested, therefore, that the pyridine nucleotide oxidation-reduction state probably exerts a more profound control on the enzymes of the citric acid cycle in vivo than the phosphate potential. However, the intramitochondrial ATP:ADP ratio could increase without this change being detected from measurements of total tissue adenine nucleotide contents, since their distribution is largely extra- and intramitochondrial (58). Control of the isocitrate dehydrogenases by the NAD+ and NADP+ oxidation-reduction state would appear to be direct. Control of citrate synthase by the NAD+ system is indirect, being mediated through the mitochondrial oxaloacetate concentration as suggested by Wieland, Weiss, and Eger-Neufeldt (59). The calculated distribution of oxaloacetate between the intra- and extramitochondrial spaces (4, 5) indicates that mitochondrial oxaloacetate concentration is below the reported $K_m$ values of 2 to 5 μM (45) for citrate synthase, whereas the mitochondrial acetyl-CoA concentrations (4) are well above the $K_m$ for acetyl-CoA (45). Since ethanol decreased both the total oxaloacetate content and the calculated concentration in the mitochondria, changes of oxaloacetate could provide an effective control at the citrate synthase step. An increased mitochondrial ATP:ADP ratio would potentiate inhibition caused by a decreased oxaloacetate concentration, since the apparent $K_m$ values for oxaloacetate and acetyl-CoA are increased by ATP (42, 45). This more complicated control could account for the observed decrease of the citrate content when ethanol was added to livers oxidizing fatty acids at a high rate. Under these conditions, the observed increase of the tissue ATP:ADP ratio suggests that an increase of mitochondrial ATP concentration could exert an auxiliary control at the citrate synthase site.

**Effect of Ethanol on β Oxidation and Ketogenesis**—Ethanol oxidation suppressed β oxidation derived from endogenous as well as exogenous fatty acids (Table VIII). Conversely, ethanol oxidation was inhibited by fatty acid oxidation. Since ethanol addition in the presence of oleate caused a further reduction of flavoproteins, it appears that the supply of reducing equivalents exceeded the rate at which they could be transmitted from the flavin to the cytochrome system. Competition may, therefore, be exerted at the level of the flavin enzymes in the respiratory chain.

Despite a 37% inhibition of β oxidation after ethanol addition in the presence of oleate, ketogenesis was stimulated, probably because of the diminished entry of acetyl-CoA into the citric acid cycle. It is of interest that the rate of ketone body production was proportional to the ratio of acetyl-CoA to CoA, but not to either acetyl-CoA alone or to the reciprocal of CoA alone. Data from rat liver in vivo (50) and isolated mitochondria (51) have shown that the rate of ketone body production is proportional to acetyl-CoA at low but not at high levels. Moreover, an inhibition of ketogenesis by CoA has been reported in a soluble system (62) and in rat liver homogenates (63, 64).

A decreased concentration of free CoA can enhance ketogenesis in different ways, depending on its subcellular location. An increase of carnitine or decrease of CoA in the cytosol will facilitate palmitoyl-CoA formation by a mass action effect on the overall palmitoylcarnitine transferase activity of mitochondria (65) with an equilibrium constant of 0.5 (66).

**Palmitoyl-CoA + carnitine ⇌ palmitoylcarnitine + CoA**

A second mitochondrial palmitoyltransferase reacts with intramitochondrial CoA and transfers the acyl group through a membrane barrier to the site of β oxidation, releasing carnitine into the extramitochondrial space (67-69). Additionally, it has been proposed (62) that intramitochondrial CoA, which allows ketogenesis by shifting the equilibrium position of the reaction

$2\text{Acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoA}$

which is highly unfavorable in the direction of acetoacetyl-CoA synthesis (70). Since the concentrations of free CoA in the intra- and extramitochondrial spaces are likely to change in the same direction during enhanced ketogenesis (4), coordinated control of a number of intermediate steps is achieved. On the other hand, increased mitochondrial acetyl-CoA concentrations will promote acetocetyl-CoA formation, and may also stimulate the formation of β-methylglyoxal-CoA from acetoacetyl-CoA and acetyl-CoA (71). The present data provide experimental support for the concept that ketogenesis increases as a function of acetyl-CoA, and decreases as a function of free CoA. Ketogenesis was enhanced during ethanol oxidation only when the ratio of acetyl-CoA to CoA increased, i.e., in the presence of exogenous fatty acids.

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