Introduction

Ethanol is a widely used drug that is consumed in large amounts for pharmacologic effects. Elimination of ethanol alters metabolism in the liver and throughout an organism. Ethanol’s effect on metabolites can influence the regulation of key pathways such as gluconeogenesis. We adopted a proton NMR-based metabolomic approach to study ethanol-induced metabolic changes in liver, blood, and brain tissue from rats exposed to either a single dose of ethanol or a chronic 4 day binge-ethanol protocol. Both acute and binge ethanol caused (i) decreased glucose, lactate, and alanine in liver and serum; (ii) increased acetate in liver and serum; and (iii) increased acetoacetate in serum. Binge-ethanol increased liver β-hydroxybutyrate and decreased betaine. Pretreatment with the antioxidant butylated hydroxytoluene (BHT) increased betaine and reduced ethyl glucuronide (EtG) in livers of binge-ethanol animals, as compared to those not pretreated with BHT. We found no change in brain metabolites after a single dose of ethanol. Unsupervised principal component (PC) analysis of spectral data from liver and serum successfully discriminated treatment groups, based largely on the biochemical differences outlined above, confirming the results of manual analysis. To explain the observed lack of gluconeogenesis following ethanol treatment and to resolve apparently discordant results from previous studies, we propose a model in which decreased hepatic alanine removes inhibition on pyruvate kinase, thus permitting a futile cycle that diverts phosphoenolpyruvate away from gluconeogenesis. This is a new mechanism that biochemically elucidates the well-known, yet unexplained, “empty calorie” phenomenon of ethanol. Reduction of EtG by pretreatment with BHT suggests that BHT and perhaps other compounds may alter the pharmacokinetics of EtG so that EtG may not always be a sensitive marker for ethanol abuse.

To understand the biochemical alterations that occur throughout the organism during ethanol ingestion, we adopted a metabolomic approach that involves the following steps: (i) simultaneously quantifying small molecule “metabolites” following exposure to ethanol, (ii) using a combination of unsupervised pattern recognition as well as manual data analysis to identify metabolic changes following ethanol exposure, and (iii) using accumulated data on biochemical reaction pathways and enzyme regulation to provide biochemical context, thus enabling formulation of generalizable conclusions and testable hypotheses. We chose to use proton NMR spectroscopy of tissue extracts and serum samples because NMR spectroscopy offers superior dynamic range, is capable of detecting many classes of molecules in the same sample, and requires minimal processing of samples prior to analysis. In 1991, Ling and Brauer used 400 MHz proton NMR spectroscopy to analyze the effect of chronic ethanol on selected liver metabolites (3). They observed an ethanol-induced decrease in liver alanine, an increase in acetate, and an increase in β-hydroxybutyrate, which was exacerbated by starvation. The present study extends these findings using (i) a 700 MHz spectrometer that affords substantial gains in resolution and sensitivity, (ii) recent literature describing assignments of various metabolites in NMR spectra, and (iii) current metabolomic methods for comprehensive spectral analysis. Numerous reports have demonstrated the utility of an NMR-based metabolomic approach with pattern recognition for the analysis of complex biochemical responses to exogenous toxins in experimental animals (4, 5) and for the classification of human patients on the basis of their biochemical

1H NMR-Based Metabolomic Analysis of Liver, Serum, and Brain Following Ethanol Administration in Rats

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Ethanol is a widely used drug that is consumed in large amounts for pharmacologic effects. Elimination of ethanol alters metabolism in the liver and throughout an organism. Ethanol’s effect on metabolites can influence the regulation of key pathways such as gluconeogenesis. We adopted a proton NMR-based metabolomic approach to study ethanol-induced metabolic changes in liver, blood, and brain tissue from rats exposed to either a single dose of ethanol or a chronic 4 day binge-ethanol protocol. Both acute and binge ethanol caused (i) decreased glucose, lactate, and alanine in liver and serum; (ii) increased acetate in liver and serum; and (iii) increased acetoacetate in serum. Binge-ethanol increased liver β-hydroxybutyrate and decreased betaine. Pretreatment with the antioxidant butylated hydroxytoluene (BHT) increased betaine and reduced ethyl glucuronide (EtG) in livers of binge-ethanol animals, as compared to those not pretreated with BHT. We found no change in brain metabolites after a single dose of ethanol. Unsupervised principal component (PC) analysis of spectral data from liver and serum successfully discriminated treatment groups, based largely on the biochemical differences outlined above, confirming the results of manual analysis. To explain the observed lack of gluconeogenesis following ethanol treatment and to resolve apparently discordant results from previous studies, we propose a model in which decreased hepatic alanine removes inhibition on pyruvate kinase, thus permitting a futile cycle that diverts phosphoenolpyruvate away from gluconeogenesis. This is a new mechanism that biochemically elucidates the well-known, yet unexplained, “empty calorie” phenomenon of ethanol. Reduction of EtG by pretreatment with BHT suggests that BHT and perhaps other compounds may alter the pharmacokinetics of EtG so that EtG may not always be a sensitive marker for ethanol abuse.
state (6). We have chosen to apply a multicompartment metabolic analysis to gain additional insight into the metabolic processes affecting the entire organism during ethanol intake. We have attempted to quantify individual metabolites in addition to submitting spectral data to unsupervised analysis, thus increasing our ability to draw specific conclusions and formulate testable hypotheses. We report here that both acute and chronic ethanol alter liver and serum metabolites but have no detectable effect on brain metabolites. In liver, both acute and chronic ethanol cause the formation of ethyl glucuronide (EtG), a process that is inhibited by the antioxidant butylated hydroxytoluene (BHT). In addition, we found that chronic 4 day binge-ethanol, but not acute ethanol, reduces betaine levels in liver, likely contributing to hepatotoxicity. We also found that acute ethanol decreases lactate and alanine in serum while increasing serum acetate and acetoacetate. Our finding that ethanol produces no increase in glucose but instead decreases alanine and lactate in both serum and liver led us to propose a new model to describe the effect of ethanol on hepatic glucose regulation in vivo. A new biochemical scheme proposed to define the mechanism by which ethanol wastes energy or creates “empty” calories (7).

Materials and Methods

Animal Handling Procedures and Sample Collection. All protocols in this study were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health regulations for the care and use of animals in research. Adult male Sprague–Dawley rats (275–325 g) were randomly selected to receive ethanol (5 g/kg, 25% w/v in water, i.g.), water (i.g.), or sucrose calorically matched to the ethanol (i.g.). Following the onset of surgical anesthesia, an abdominal incision exposed the liver and inferior vena cava. A blood sample (2–4 mL) was collected from the inferior vena cava and allowed to clot on ice. Next, each animal was decapitated and the skull was immediately immersed in liquid nitrogen with agitation in the liquid to hasten the freezing process, while a small piece of liver (1–2 g) was quickly removed, wrapped in aluminum foil, and frozen in liquid nitrogen. Clotted blood was centrifuged, and serum was removed and stored along with liver and skull samples at −80 °C until further analysis.

After the experiment, it came to our attention that the vehicle for the pentobarbital contained 50% water, 40% propylene glycol, and 10% ethanol. Nevertheless, because this dose (~0.2 g/kg) was (i) substantially smaller than the 5 g/kg dose given to the ethanol-treated group, (ii) present only for 5 min before sacrifice, and (iii) would, if anything, tend to minimize differences between groups, creating a more stringent challenge for the metabolomic approach, we deemed it appropriate to proceed with the analysis.

In a separate experiment, rats were treated with a modified 4 day binge-ethanol protocol as described previously (8). This protocol produced physical dependence to ethanol (9) and induced neuropathology modeling that has been found in human alcoholics (8). Prior to the start of experiments, half of the animals were treated with the antioxidant BHT (10) (120 mg/kg/day in corn oil, i.g.) for 4 days, which has been found to protect against alcohol-induced brain damage (11). BHT control animals received only corn oil vehicle. A 15% (w/v) ethanol Reitz high-fat diet was used with an appropriate isocaloric control. Male Sprague–Dawley rats (250–300 g) were given an initial dose (5 g/kg, 25% w/v, in a vehicle of nutritionally complete diet) with subsequent doses determined using a six-point behavior scale (9). Control animals received calories (from maltodextrin) equal to the average of all ethanol-treated animals. All animals had free access to water throughout the experiment. The blood ethanol concentration was measured using electrochemical detection of an enzymatic reaction with an Analox Instruments model GM-7 analyzer (Analox Instru-
dimensional) data, with different treatment groups segregating themselves in the new PC coordinates (18). Moreover, analysis of the PC vectors afforded the opportunity to determine which spectral regions contributed to the separation of spectra from different treatment groups, thereby providing insight into the biochemical differences and possible mechanisms for toxicity (4).

Statistical Analysis. In all charts, data were plotted as means ± standard errors of the mean, and the number of animals in each group was indicated. When comparing means between different treatment groups, a t test (two tailed, unequal variance) was used to assess statistical significance. A p value of less than 0.05 was deemed significant. We have opted to present p values without correction for multiple comparisons for the following reasons. The first objection concerned the data itself: Many of the metabolites quantified in this report were linked via their participation in known biochemical networks, and therefore, concentrations of many were not statistically independent; the commonly used Bonferroni correction was therefore inappropriately conservative (19). The second objection concerned study design: By their nature (i.e., large number of measured parameters, comparatively small number of samples), metabolomic studies were designed to generate hypotheses rather than to test specific hypotheses. For this type of study, an optimal multiplicity correction method has not been universally accepted (20), and the approach of presenting the uncorrected p values has been advocated instead (21).

Biochemical Interpretation. To the extent possible, biochemical perturbations due to ethanol exposure were interpreted within the framework of the known chemical reactions of intermediary metabolism. In addition to standard texts, the KEGG database (22) was a useful resource for ascertaining metabolic relationships between various molecules.

Results

1H Spectroscopic Analysis of Liver. Ethanol is a unique drug that provides calories when metabolized. There were two controls, one with water and a second control for ethanol calories using a sucrose solution containing ethanol-equivalent calories. Comparing water controls to sucrose controls, the sucrose control caused a 65% decrease in hepatic β-hydroxybutyrate (p = 0.014), a 2.8-fold increase in lactate (p = 0.0092), a 2.7-fold increase in alanine (p = 0.0052), a 1.4-fold increase in acetate (p = 0.041), a 2.3-fold increase in glucose (p = 0.0020), and a nonsignificant trend toward increased succinate. Ethanol-induced perturbations in several hepatic metabolites were evident in 1H NMR spectra of extracts (Figure 1). In comparison to water controls, single-dose ethanol animals showed a 72% decrease in alanine (p = 0.038), a 1.7-fold increase in acetate (p = 0.011), and nonsignificant trends toward decreased lactate and increased β-hydroxybutyrate (Figure 2). As compared to sucrose controls, the single-dose ethanol animals exhibited a decrease in alanine of 90% (p = 0.0016), a lactate decrease of 80% (p = 0.0040), and a glucose decrease of 60% (p = 0.0016), while β-hydroxybutyrate increased 5.1-fold (p = 0.011). Hepatic acetate was comparable between ethanol and sucrose groups, and succinate was not affected by ethanol or sucrose treatment. These comparisons suggest that providing energy to the liver through ethanol or sucrose increases acetate, whereas ethanol uniquely decreases alanine and lactate without increasing glucose.

Turning our attention to the binge-ethanol groups as compared to binge-diet controls discussed above, binge-ethanol treatment decreased hepatic alanine 92% (p = 0.00018), lactate 89% (p = 0.000083), and succinate 60% (p = 0.000063), whereas it increased acetate 9-fold (p = 0.00028) and β-hydroxybutyrate 15-fold (p = 0.023). Overall, the binge control and binge control + BHT groups were similar; the BHT pretreated group showed modest decreases in lactate 31% (p = 0.043) and alanine 41% (p = 0.019). For the endogenous metabolites shown in Figure 2, binge-ethanol and binge-ethanol + BHT groups showed no significant differences, indicating a lack of effect of BHT pretreatment on ethanol’s perturbation of these metabolites.

Comparing the binge and single-dose groups (Figure 2), the most striking difference is that succinate levels were lower in the single-dose animals than the binge animals (e.g., comparing binge control to single-dose water shows a 94% decrease, p = 0.000069). These differences were most likely related to differences between the liquid diet fed to binge animals and the standard chow given to the single-dose animals. Taking sucrose treatment and binge-control diets to be indicative of the fed state, it appears that overnight fasting also decreases lactate and alanine but not as much as ethanol. As compared to the fed state (i.e., sucrose or binge control), ethanol decreases hepatic glucose levels. Thus, in comparison to the fed state, both acute and binge-ethanol decreased lactate, alanine, and glucose, with somewhat larger decreases in the 4 day binge treatment. Additionally, acetate levels were increased in water- and sucrose-treated animals relative to binge controls, and the water-treated animals had lower hepatic alanine 77% (p = 0.000065) and lactate 75% (p = 0.000093). These findings most likely reflect the fact that all single-dose animals (including sucrose and water groups) received a small amount of ethanol in the vehicle of the pentobarbital anesthetic (see methods). In summary, both acute and binge-ethanol decrease lactate and alanine and increase acetate and β-hydroxybutyrate.

Analysis of the liver spectra revealed a triplet at 1.24 ppm in both acute and binge-ethanol-treated animals but not in controls (Figure 1). The possibility that this signal originated from residual ethanol (ca. 1.18 ppm) that had not evaporated during lyophilization (as suggested in 3) was ruled out due to incorrect chemical shift, and the signal was assigned to EtG as described in ref 11 and was quantified (Figure 3). EtG is undetectable in livers from animals that received no ethanol, but within 3 h of a large dose of ethanol, hepatic levels rise to the 2 mmol/kg range and remain elevated during the 4 day binge. Interestingly, pretreatment with BHT caused a 90% decrease (p = 0.008) in the formation of EtG during 4 days of binge-ethanol treatment. Thus, EtG is a unique metabolite found only with ethanol administration and reduced by BHT pretreatment.

A strong singlet at 3.28 ppm in liver spectra (Figure 1a) was investigated. The spectra were analyzed after addition of betaine and trimethylamine N-oxide (TMAO), two metabolites commonly assigned to signals in this region (23). The consistent finding was that the large singlet in our original spectra was due to betaine and that the signal from TMAO, if present, was at least an order of magnitude smaller (Figure 4). Having identified the signal as originating from betaine, the peak was quantified and results were compared across treatment groups. Betaine did not appear to be altered by a single dose of ethanol, but both binge-ethanol and binge-ethanol + BHT showed a trend of decreased betaine, with the binge-ethanol + BHT group significantly lower than the binge-control + BHT group (p = 0.0033) but not significantly different from the binge-control group that received no BHT (Figure 5). Thus, BHT seems to elevate liver betaine, a single dose of ethanol has no effect, and binge-ethanol treatment showed a trend toward a decrease in betaine.

Homocysteine is difficult to detect by 1H NMR for two reasons: First, the partially resolved γ-methylene (δ 2.64) resonance is under-represented in the NMR spectrum due to splitting of its two protons from its β-methylene (δ 2.13) into an ABXY multiplet (1H NMR spectrum obtained from the
human metabolome database, http://www.hmdb.ca/), while its transmethylation reaction partner, betaine, is over-represented by its nine protons from the trimethylamine, which combine to a singlet. Therefore, homocysteine is effectively 25-fold under-represented (i.e., assuming an ABCD pattern no higher order coupling) relative to betaine. In addition, these two resonances of homocysteine partially overlap with $\gamma$-methylene of $\gamma$-glutamylglutathione and $\gamma$-methylene of glutamate. Therefore, homocysteine was not easily detectable by $^1$H NMR and likely would not contribute to the loading plots.

**PC Analysis of Liver NMR Spectra.** After manually selecting spectral regions with identifiable signals for quantification to obtain the results described above, the data were submitted to unsupervised PC analysis to examine spectral data in a completely unbiased manner. This approach provided us with a purely statistical assessment of the most important biochemical differences between our treatment groups. First, PCA was performed on data from all seven groups of liver extracts (Figure 9). Separation along PC1 (which explained 46.9% of the variance in this data set) differentiated primarily single-dose from binge groups, irrespective of ethanol treatment (data not shown). However, binge-ethanol groups (with and without BHT) separated from others along PC2 (16.7% of variance) and PC3 (15.6% of variance), with the single-dose ethanol group showing some separation along PC3. Thus, positive values of PC2 and PC3 were associated with ethanol-induced metabolic changes. Loadings plots for PC2 and PC3 (Figure 9c,d) indicate that choline-containing compounds ($\delta$ 3.20–3.24), betaine ($\delta$ 3.24–3.28, $\delta$ 3.92–3.96), lactate ($\delta$ 1.32–1.36, $\delta$ 4.08–4.16), alanine ($\delta$ 3.20–3.24), EtG ($\delta$ 1.20–1.24) were among the largest contributors to separation of the binge-ethanol-treated animals. Interestingly, the energy metabolite, adenine nucleotide ($\delta$ 8.24–8.28 (adenine C2); $\delta$ 6.12–6.16, 5.96–6.00 (ribose)), also was a minor contributor to PC2 and PC3. On the basis of the loading plots, liver ethanol was associated with increased levels of EtG, acetate, and choline compounds and depletion of alanine, lactate, glucose, and glycogen.
For an additional level of analysis, we performed PCA on the binge and single-dose groups separately (Figure 10). For binge animals (Figure 10a), there was relative separation of the control diet vs control + BHT groups, whereas the binge-ethanol and binge-ethanol + BHT groups comingle in PC space. Loadings plots (Figure 10c,d) indicate that choline-containing compounds and betaine made the largest contribution to PC1, with lactate, glutamate ($\delta 2.12–2.20$), and EtG also contributing. These signals also contributed to PC2, along with the spectral regions containing unresolved glucose, glycogen, and amino acids ($\delta 3.3–4.0$), as well as resolved glucose ($\delta 5.24$) and glycogen ($\delta 5.42$). Single-dose groups show a large separation in the scores plot (Figure 10b), with PC1 differentiating ethanol from sucrose and PC2 separating water from the other groups. The loading plots (Figure 10e,f) reveal that once again betaine, lactate, alanine, EtG, and sugars (glucose at 5.24 ppm and glycogen at 5.42 ppm) were significant contributors to the PCs. In comparison to the binge animals, EtG made a relatively larger contribution to both PC1 and PC2 in the single-dose group, indicating that formation of this unique compound is one of the earliest markers of hepatic ethanol metabolism.
**1H Spectroscopic Analysis of Serum.**

In comparison to animals that received sucrose, ethanol decreased lactate 76% \((p = 0.040)\), decreased alanine 62% \((p = 0.0015)\), decreased glucose 39% \((p = 0.0021)\), but increased acetate 28-fold \((p = 0.000079)\). In the ethanol-treated animals, the serum ethanol concentration 3 h after dosing was 77 ± 6 mM. Ethanol (6 ± 2 mM) and propylene glycol were discovered in a 1:4 ratio in the serum of sucrose- and water-treated animals since these were components of the vehicle for the pentobarbital anesthetic (see Materials and Methods). Because of spectral overlap with the very large signal from ethanol, \(\beta\)-hydroxybutyrate could not be quantified in serum.

**PC Analysis of Serum NMR Spectra.** Single-dose animals that received ethanol separated dramatically from water and sucrose groups along PC1 (Figure 11a). Water and sucrose groups show separation along the PC2 axis. Because of the decision not to use relaxation editing (24) to suppress signals from proteolipids, the spectra contain many broad components, especially in the 0.5–3.0 ppm region, and these are reflected in the loadings plots (Figure 11c,d). Loadings plots revealed that PC1 contained signals from lactate, glucose \((\delta 3.20–3.28, \delta 3.44–3.48, \delta 3.52–3.56, \delta 3.88–3.92, \delta 5.20–5.28)\), and two composite acetyl signals from \(\alpha_1\)-acid glycoprotein (AGP) \((\delta 2.04–2.08, \delta 2.12–2.16)\) \((13)\); with respect to water and sucrose controls, ethanol-treated animals showed decreases in all of these compounds (negative PC1 values). PC2 separates groups based on an inverse relationship between lactate and Nac1,2, with an additional contribution from the signal at 0.84–0.88. On this basis, sucrose-treated animals have a low Lac/Nac1,2 ratio (positive PC2 values) whereas water-treated animals have a low Lac/Nac1,2 ratio (negative PC2 values).

**1H NMR spectra of serum revealed changes in many metabolites after a single dose of ethanol or sucrose, as compared to water (Figures 6 and 7).** In comparison to water, sucrose treatment decreased acetate 89% \((p = 0.00092)\), increased glucose 18-fold \((p = 0.00020)\), and resulted in nonsignificant trends toward increased lactate and acetate. Ethanol, in comparison to water, decreased lactate 57% \((p = 0.0073)\), decreased alanine 62% \((p = 0.00066)\), increased acetate 2-fold \((p = 0.0062)\), increased acetate 3-fold \((p = 0.000074)\), and had no effect on glucose. In singe-dose animals that received ethanol separated dramatically from water and sucrose groups along PC1 (Figure 11a). Water and sucrose groups show separation along the PC2 axis. Because of the decision not to use relaxation editing (24) to suppress signals from proteolipids, the spectra contain many broad components, especially in the 0.5–3.0 ppm region, and these are reflected in the loadings plots (Figure 11c,d). Loadings plots revealed that PC1 contained signals from lactate, glucose \((\delta 3.20–3.28, \delta 3.44–3.48, \delta 3.52–3.56, \delta 3.88–3.92, \delta 5.20–5.28)\), and two composite acetyl signals from \(\alpha_1\)-acid glycoprotein (AGP) \((\delta 2.04–2.08, \delta 2.12–2.16)\) \((13)\); with respect to water and sucrose controls, ethanol-treated animals showed decreases in all of these compounds (negative PC1 values). PC2 separates groups based on an inverse relationship between lactate and Nac1,2, with an additional contribution from the signal at 0.84–0.88. On this basis, sucrose-treated animals appear to have a large Lac/Nac1,2 ratio (positive PC2 values) whereas water-treated animals have a low Lac/Nac1,2 ratio (negative PC2 values).

**Figure 6.** Proton spectra (700 MHz) of serum collected from a control animal (a), 3 h after a single dose of ethanol (b), and 3 h after dosing with sucrose (c). Large signals from ethanol are evident in the ethanol-treated animals, so large in fact that the satellite peaks due to natural abundance (1.1%) \(^{13}\)C are visible. Ethanol decreases lactate and alanine signals, while increasing acetoacetate and acetate, as compared to dosing with water. As discussed in the Materials and Methods, the sucrose and water control groups were also inadvertently administered a small dose of ethanol (<0.2 g/kg, i.p.) in the vehicle for the pentobarbital anesthetic, and signals from ethanol are seen in parts a and c. The key is the same as in Figure 1, with the addition of Aca, acetoacetate; Cit, citrate; Nac1/Nac2, composite acetyl signals from \(\alpha_1\)-acid glycoprotein; and S1/S2, \(^{13}\)C satellites of ethanol.

**Figure 7.** Effect on selected serum metabolites 3 h after i.g. administration of ethanol (5 k/kg), water, or sucrose. Lactate and alanine decreased at least 2-fold in ethanol-treated animals in comparison to animals treated with water or sucrose. Acetate increased 2.1-fold in ethanol-treated animals vs water-treated animals but not in comparison to sucrose-treated animals. It is likely that inadvertent administration of ethanol (in the vehicle of the pentobarbital solution; see the Materials and Methods) to all animals just before sacrifice gave falsely high acetate levels in sucrose and water groups. Serum glucose was 1.6-fold lower in ethanol-treated animals than in sucrose controls. Key: *, \(p < 0.05\); **, \(p < 0.005\); and ***, \(p < 0.0005\).

1\(^1\)H NMR spectra of serum revealed changes in many metabolites after a single dose of ethanol or sucrose, as compared to water (Figures 6 and 7). In comparison to water, sucrose treatment decreased acetate 89% \((p = 0.00092)\), increased glucose 18-fold \((p = 0.00020)\), and resulted in nonsignificant trends toward increased lactate and acetate. Ethanol, in comparison to water, decreased lactate 57% \((p = 0.0073)\), decreased alanine 62% \((p = 0.00066)\), increased acetate 2-fold \((p = 0.0062)\), increased acetate 3-fold \((p = 0.000074)\), and had no effect on glucose. In

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1 Abbreviations: AGP, \(\alpha_1\)-acid glycoprotein; BHMT, betaine-homocysteine S-methyltransferase; BHT, butylated hydroxytoluene; ETG, ethyl glucuronide; NAD, nicotinamide adenine dinucleotide; PEPCK, phosphoenolpyruvate carboxykinase; PC, principal component; PK, pyruvate kinase; TMAO, trimethylamine N-oxide; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid; UGTs, UDP glucuronosyltransferases.
Few changes were evident in the signals from brain metabolites (Figure 8). Although signals were well-resolved and many metabolites were quantified, no clear trends in metabolite concentrations were detectable 3 h after a single dose of ethanol.

**PC Analysis of Brain NMR Spectra.** PC analysis of brain spectra revealed incomplete separation of the ethanol, water, and sucrose groups in the scores plot (Figure 11b), although some trends were evident. The mean PC2 value for the ethanol group was lower than either of the other two groups. The water-treated group was tightly clustered in PC1 but showed a large spread in PC2. The loadings plots (Figure 11e,f) reveal that lactate was the largest contributor to PC1 (which explained 45.9% of the variance), followed by creatine + phosphocreatine (δ 3.96–4.00). PC2 showed two dispersive (“one up, one down”) pairs indicative of signals (glutamate + glutamine at δ 3.80 and taurine + myo-inositol at δ 3.32) whose frequency drifted at least one bin width (0.04 ppm, 28 Hz) across the data set. Combining these pairs of buckets into a single bin (width 0.08 ppm) and repeating PC analysis did not yield appreciable improvement in the separation of the groups in the scores plot (data not shown). These results suggest that intragroup variability in metabolite concentrations was on the same order as the variance between treatment groups.

**Discussion**

**Energy Metabolites in Liver and Serum.** Our findings shed light on the effects of ethanol on gluconeogenesis and the manner in which lactate and alanine participate in these effects. We found that both acute and binge-ethanol decreased lactate and alanine in rat liver and in serum, consistent with previous studies (3). However, there is not a consensus on the effect of ethanol on glucose regulation. Contradictory results were reported in early studies in perfused mouse livers quantifying production of glucose using absolute concentration as a measure. One reported that ethanol stimulates gluconeogenesis from alanine and increases the consumption of lactate (25), and another in the same year by Krebs himself reported that ethanol inhibits gluconeogenesis (26). For isotopic studies measuring flux in perfused mouse livers, acute ethanol stimulates hepatic utilization of [3,13C]alanine for gluconeogenesis (27). More recently, Mokada et al. reported that incorporation of 14C from lactate into glucose in perfused rat liver was inhibited up to 80% by ethanol (28). Our data are consistent with the consumption of lactate and alanine; however, we find no evidence of increased glucose production following acute or binge-ethanol administration (Figures 2 and 7). Our data suggest decreased glucose and glycogen levels after binge-ethanol administration and no increase in hepatic glucose or glycogen following a single dose of ethanol. Ethanol has long been known to inhibit gluconeogenesis (28).

Figure 12 shows a possible mechanism to explain these apparently discordant results: Elevated NADH initially stimulates gluconeogenesis, flux through pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK) increases, and gluconeogenic precursors (lactate and alanine) replenish the pyruvate that is consumed. Quickly, however, alanine is depleted, and decreased alanine levels remove the inhibition of pyruvate kinase (PK) (29), leading to a futile cycle from pyruvate to oxaloacetate to phosphoenolpyruvate (PEP) and then back to pyruvate. This cycle consumes two ATP, while producing just one ATP; therefore, each cycle consumes one ATP. Interestingly, ATP/ADP was one of the biomarkers identified in PC1 and PC2 loading plots (Figure 10), decreasing in the acute ethanol group (Figure 10e) but increasing in the chronic binge ethanol group (Figure 10d). A recent study using 13C acetate in rat brain found that pyruvate recycling occurs in peripheral tissue, likely liver (30), supporting our proposed model. Pyruvate cycling can alleviate increases in mitochondrial membrane potential, which increases with ethanol exposure due to the production of NADH during acetaldehyde oxidation (31). This is achieved by regeneration of ADP, which is the substrate...
for mitochondrial ATP synthase, which consumes three protons per ATP generation, thereby reducing inner mitochondrial membrane potential.

One may explain the apparently contradictory results of the studies mentioned above by the observation that decreased intracellular alanine during ethanol oxidation is critically important in the regulation of gluconeogenesis. Whereas alanine is rapidly depleted during ethanol oxidation in vivo (Figure 2), Cohen et al. (27) supplied alanine in the perfusate so that intracellular alanine was maintained even during ethanol oxidation. Without a decrease in intracellular alanine, PK remained inhibited, and PEP was used for gluconeogenesis, avoiding the futile cycle and producing $^{13}$C-glucose from $^{13}$C-alanine. When lactate rather than alanine was supplied in the perfusate (28), alanine was depleted (as it is in vivo), and gluconeogenesis was short-circuited by increased PK activity. Although our proposed mechanism explains the discordant findings, additional studies with labeled tracers are required to prove the operation of a futile cycle during ethanol metabolism. Our studies suggest that in vivo ethanol does not stimulate gluconeogenesis in liver due to depletion of alanine. Also, unlike our study using intact rats, all of the studies mentioned above used perfused liver systems; although an extremely powerful tissue model, it can produce nonphysiological conditions of unlimited substrate availability.

Our study found that ethanol decreased liver and serum lactate, whereas an entirely different perspective on hepatic lactate metabolism during ethanol exposure has been previously suggested. Blood lactate in human volunteers was reported to increase following ethanol administration, purportedly because the elevated NADH/NAD ratio stimulated conversion of pyruvate to lactate in hepatocytes, to regenerate reduced NAD cofactors in the cytosol (32) where alcohol dehydrogenase is active (1). According to this view, the lactate produced in hepatocytes would be released into the blood where hyperlac-
tacemia would inhibit excretion of uric acid in the kidney, thus explaining the association between alcohol consumption and gout (32, 33). Despite the conceptual appeal of this explanation, it is at variance with our finding of decreased lactate in both liver and blood after both acute and chronic ethanol exposure. We note that the original finding of increased blood lactate after ethanol infusion (32) was based on a colorimetric assay that relied upon the conversion of lactate to acetaldehyde by heating in concentrated sulfuric acid (34). Given that blood ethanol levels were in the 200–300 mg/dL range (44–65 mM) while blood lactate was 0.7 mg/dL (0.8 mM) (32), even as little as 1% of this ethanol converted to acetaldehyde during the procedure would cause falsely elevated estimates of serum lactate levels. We believe that this flaw in the original assay is the most likely explanation for the discordant findings. We believe that ethanol decreases lactate in both liver and serum due to futile cycling of pyruvate in liver (Figure 12).

The trends toward increased acetate in liver and serum (Figures 2 and 7) are consistent with the oxidation of ethanol to acetate in the liver, and the release of 90% or more of the ethanol-derived acetate into the blood (2). Animals in the 4 day binge group showed liver acetate concentrations that were no greater than the single-dose animals, suggesting either (i) no increase in the rate of ethanol oxidation to acetate or (ii) simultaneous increase in acetate formation and acetate efflux from liver into blood. Unfortunately, the single-dose control

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Figure 10. PC analysis of liver samples for binge-ethanol and binge control groups (a), separately from single dose groups (b). By separating the analysis in this way, the variance due to ethanol treatment is separated from differences in diet and other factors that obscured the effect of ethanol treatment in Figure 9. Loading plots indicating the spectral regions that contribute to separation of various treatment groups: (c) binge PC1, (d) binge PC2, (e) single dose PC1, and (f) single dose PC2. The key is the same as in Figures 1 and 9 except that the resonances at 8.24, 6.12, and 5.96 ppm are due to adenine nucleotide.
animals inadvertently received ethanol as a component of the vehicle for the pentobarbital anesthetic and therefore displayed blood acetate levels greater than the 0.2–0.3 mM expected in the absence of ethanol (35). Nevertheless, the trends toward increased acetate in the serum of single-dose ethanol-treated animals are still evident (Figure 7). In the liver, if the binge-control values (Figure 2) are taken as representative of the baseline state (since these animals received no ethanol due to anesthetic), the effect of a single dose is clearly to increase acetate several-fold. Increased serum acetate could affect metabolism in a number of tissues and indicates that although the liver metabolizes the majority of a dose of ethanol, the resulting acetate could alter metabolism throughout the organism.

The effect of ethanol on ketone body formation can be seen in the increase in β-hydroxybutyrate in the liver (Figure 2) and acetoacetate in the serum (Figure 7). It is likely that these molecules represent a means for the liver to both dispose of the surfeit of ethanol-derived acetate and to regenerate reduced cofactors. In a reaction catalyzed by acetyl-CoA C-acetyltransferase (EC 2.3.1.9), two molecules of acetyl-CoA condense to form acetoacetyl-CoA, which is converted to acetoacetate via 3-ketoacid CoA-transferase (EC 2.8.3.5) (22). Subsequently, acetoacetate can be converted to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), regenerating one molecule of NAD from NADH and thus facilitating continued oxidation of ethanol. The fact that acetoacetate was not detectable in liver spectra suggests that this molecule is transported into the blood. Because both a single dose of ethanol and the 4 day binge treatment produced a similar increase in β-hydroxybutyrate, there does not appear to be an induction of

![Figure 11. PC analysis of single dose serum (a) and brain (b). Serum shows clear separation of alcohol-treated animals (triangles) from water (circles) and sucrose-treated animals (squares) along PC1, with some separation of water and sucrose-treated animals along PC2. Brain shows some separation of groups along PC1, but PC2 appears to be less informative. Loadings plots for serum PC1 (c) and PC2 (d) and brain PC1 (e) and PC2 (f) indicate which spectral regions contribute to the classification of various groups.](image-url)
the pathways metabolizing ethanol to β-hydroxybutyrate in the time span studied in these experiments.

Betaine Metabolism and Transmethylation. Previous studies have found that betaine, an important precursor of S-adenosylmethionine (SAM), is decreased by chronic ethanol administration in rats due to increased activity of betaine-homocysteine S-methyltransferase (BHMT, EC 2.1.1.5), which catalyzes the formation of methionine from betaine and homocysteine (36). Moreover, dietary supplementation with betaine during chronic ethanol feeding prevents the formation of fatty liver (37), and betaine protects rat hepatocytes (38) and mouse liver (39) and hepatocytes (40) from ethanol-induced apoptosis. Ethanol-induced apoptosis has been proposed to be caused by elevated levels of homocysteine that cause endoplasmic reticulum stress response (41). Although clinical serum levels can reach as high as 120 μM homocysteine (42), it was under-represented and not well-resolved in the 1H NMR (see the Materials and Methods for an explanation) and thus was not identified in the PCA loading plot (Figures 9 and 10). Treatment of control animals with BHT increased betaine (Figure 5), presumably by altering the flux of homocysteine through BHMT, although the precise mechanism remains unclear. On the other hand, it appears that during binge-ethanol exposure, BHT does not prevent a decrease in hepatic betaine (Figure 5). To our knowledge, this is the first finding of decreased hepatic betaine after only 4 days of ethanol intake. This finding suggests that brief, intense, continuous exposure to ethanol alters metabolites involved in methylation and may contribute to alcoholic liver injury.

α1-Acid Glycoprotein in Serum. Results of PC analysis of serum samples suggested decreased signals from AGP in serum of animals acutely dosed with ethanol. AGP exists as a serum glycoprotein where it binds basic drug molecules, but it also plays other roles, most notably as a modulator of immune function (43). Our finding of decreased AGP with ethanol treatment is consistent with a previous report that suggested that reduced numbers of AGP-bearing lymphocytes in chronic alcoholics may contribute to poor immune function in these individuals (44). On the other hand, AGP is at once both a stimulator of TNFα secretion by macrophages as well as an inhibitor of TNFα-induced apoptosis (43). Moreover, AGP can bind endotoxin and prevent toxic shock in rats (43). Thus, ethanol-induced decreases in AGP may contribute to ethanol-induced liver injury via an immune mechanism stimulated by endotoxin released when ethanol lyses gut flora (45). In summary, ethanol decreases serum AGP, and this molecule may be involved with ethanol-induced pathology via several interesting mechanisms.

Brain Metabolites. Given the large number of well-resolved peaks from a large number of molecules in the brain spectra, it would seem an ideal data set for metabolomic analysis. The relative lack of effect of ethanol on endogenous brain metabolites is somewhat surprising, but one must keep in mind that ethanol’s well-documented effects on the release of various amino acids from neurons and astrocytes in the brain will not be detected in whole-brain extracts. The only effects that we can observe in this study are those that change the whole-brain concentration of a given molecule: net synthesis, catabolism, or transport. Previous studies using in vivo proton NMR spectroscopy (MRS) after a single dose of ethanol failed to detect changes in endogenous brain metabolites in rhesus monkeys (46) and humans (47). Following 16 weeks of ethanol treatment in rats, phosphocholine increased 1.2-fold, and after 60 weeks, glycerophosphocholine decreased 1.5-fold (48), suggesting that the effect of chronic ethanol exposure varies over time. Other investigators found a 19% decrease in the ratio of myoinositol/total creatine after 8 weeks of ethanol treatment in rats (49). Perhaps a more pertinent measure than the effect of ethanol on concentrations of brain metabolites is the effect of ethanol on various fluxes of metabolites in brain. Brain metabolism is known to be compartmentalized in astrocytes and neurons, with separate TCA cycles operating in each compartment (50). Acetate is a substrate for the astrocytic compartment (51), and during chronic ethanol exposure, it may disrupt the normal metabolic coupling between neurons and astrocytes required for neurotransmission (52). Studies are currently underway to address this point. In summary, a single dose of ethanol did not significantly alter the whole-brain concentration of the metabolites measured in this study.

EtG. Previous studies have found that ethanol treatment can cause hepatic formation of EtG (53). Reports have focused on the determination of EtG using sensitive techniques such as ELISA (54) and GC-MS (53) to achieve low detection thresholds; however, quantification via NMR has not been demonstrated to date (11). The fact that hepatic EtG reached near maximal levels (2 mmol/kg) within 3 h of ethanol consumption (Figure 3) while the time constant for elimination is on the order of days (55) suggests either a significant barrier to the release of EtG from the liver into the blood or a significant enterohepatic circulation. Our studies suggest that EtG is elevated by the first dose of ethanol and remains elevated for days during binge-ethanol treatment. Comparing PC analysis of single-dose vs binge-ethanol-treated liver spectra, EtG made a relatively larger
contribution to the loadings plots of the acute animals, suggesting that EtG is one of the most significant early changes in liver metabolites during ethanol exposure.

Interestingly, BHT reduced the formation of EtG (Figure 3). To our knowledge, this is the first report of decreased EtG in vivo due to consumption of a compound found in normal diets. EtG is synthesized in microsomes through the action of UDP glucuronyltransferases (UGTs, E.C. 2.4.1.17); the reaction proceeds at a slow rate and is inhibited by bilirubin and other inhibitors of UGTs (56). We know that 35% of BHT is eliminated via glucuronidation (10), and thus, it is logical that BHT is a substrate for UGTs and may act as a competitive inhibitor of these enzymes, resulting in decreased formation of EtG. The finding that BHT pretreatment (and presumably pretreatment with other inhibitors of UGTs) can decrease the formation of EtG has important implications for the use of EtG as a biomarker for ethanol abuse. While this phenomenon would not produce false-positive results, it may diminish the value of negative EtG test results in ruling out recent ethanol use (57–59). Additional studies, particularly in humans, will be required to clarify whether dietary supplements and pharmaceuticals can significantly alter EtG levels following ethanol abuse.

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