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EFFECT OF CHRONIC ALCOHOL INGESTION ON HEPATIC FOLATE DISTRIBUTION IN THE RAT

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Abstract—The mechanism by which ethanol impairs folate metabolism remains uncertain. In the present study, we used our new technique (affinity/HPLC) for folate analysis to study the effect of chronic alcohol ingestion on the content and distribution of folates in livers. Twelve male Sprague–Dawley rats (180 g) were divided into two groups, and fed for 4 weeks with Lieber–DeCarli semi-liquid isocaloric diets, with and without 5% ethanol. Livers were extracted in boiling, pH 9.3 borate buffers containing ascorbate/dithioerythritol. Folates in the supernatant fractions were purified by affinity chromatography and analyzed using ion pair high performance liquid chromatography. The data obtained showed that hepatic folate distribution in alcohol-treated rats differed from that of control animals in two ways. Livers from the ethanol-fed rats, when compared with those from control rats, exhibited increases in the percent concentrations of methylated tetrahydrofolates (21.46 ± 2.21 vs 14.8 ± 1.23), decreases in the percent concentrations of formylated tetrahydrofolates (25.62 ± 4.02 vs 46.18 ± 2.65) and higher concentrations of unsubstituted tetrahydrofolates (52.91 ± 3.84 vs 38.88 ± 2.50). In addition, alcohol ingestion was associated with longer glutamate chains of the folate molecules, characterized by lower relative concentrations of pentaglutamyl folates (29 vs 48%), and higher relative concentrations of hexa- and heptaglutamyl folates (55 vs 46% and 15 vs 6%) when compared with controls. The data are discussed in relation to the possibility that alcohol exerts its effect through: (1) inhibition of B12-dependent methyl transfer from methyltetrahydrofolate to homocysteine; (2) diversion of formylated tetrahydrofolates toward serine synthesis; and (3) interaction of acetaldehyde with tetrahydrofolates, thereby interfering with folate coenzyme metabolism.

Key words: ethanol; acetaldehyde; folate; folate analysis; rats

The deleterious effect of alcoholism on folate status has been known for some time [1–3]; however, the mechanism of this effect remains unknown. Poor dietary intake, intestinal folate malabsorption or altered hepatic folate metabolism [4–7] have been proposed as contributing to this effect of alcohol. There is a consensus among investigators that acute alcohol ingestion in both humans and laboratory animals causes a decrease in the plasma folate level [8–10] while enhancing urinary folate excretion [11–14]. There are, however, inconsistencies as to the nature as well as to the concentration of folates in the liver of alcohol-fed rats when compared with those in the liver of control rats. Prolonged alcohol ingestion has been reported to be associated with a decrease in hepatic folate concentration in monkeys [14], while others have reported that in rats alcohol is associated with an increase in hepatic folate concentration [9, 15, 16]. Brown *et al.* [17] reported that alcohol ingestion causes a decrease in folate polyglutamylation in the liver. Others reported the opposite [9, 15, 16].

Many of the studies on the effect of alcohol on hepatic folates have relied on the analysis of radioactivity distribution in liver following the injection of [^{3}H]folates into animals. These analyses, for the most part, were conducted within the first 24 hr after the administration of the label. This approach provided valuable information, particularly in regard to the effects of alcohol on the fate of newly assimilated folate. There was no certainty, however, as to whether the observed alcohol-related differences in radioactive folate distribution reflected alcohol-related differences in endogenous folates. The indications are that a period of 24 hr is not sufficient for the complete equilibration of administered labeled folate with endogenous folates in the liver [18]. Our goal in the present study was to determine the effect of chronic alcohol feeding on the content and distribution of endogenous folates in rat liver. Endogenous folates were determined using a method recently developed in our laboratory, which combines affinity chromatography and ion pair liquid chromatography (Affinity/HPLC method), for the analysis of folate content and distribution [19, 20]. We were particularly interested in obtaining evidence to support the findings by Barak *et al.* [21–24], who showed that alcohol ingestion is associated with a 50% decrease in hepatic methyltetrahydrofolate: homocysteine methyltransferase activity, whereas

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that of betaine:homocysteine methyltransferase increases by more than 50%.

MATERIALS AND METHODS

[3′,5′,7,9-^{3}H]Folic acid ([^{3}H]folic acid, 40 Ci/mmol) was purchased from Moravek Biochemicals Inc (Brea, CA). Folic acid casei medium (ATCC 7469) was obtained from Difco Laboratories (Detroit, MI); sodium ascorbate, bis-Tris, dithioerythritol, 2-mercaptoethanol and tetrabutylammonium phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO), and acetonitrile was obtained from Fischer (Fairlawn, NJ).

Twelve male Sprague–Dawley rats (180 g) were randomly assigned to two equal groups and housed individually. Group 1 (C) was the control group which was fed the Lieber–DeCarli semi-liquid diet [25]. Group 2 (A) was fed the Lieber–DeCarli semi-liquid ethanol diet (5% alcohol). All diets contained folic acid at the same concentration of 2 mg/kg. Feeding was controlled to ensure isocaloric intake of all rats in the two groups. Rats were maintained on this dietary regimen for 4 weeks after which they were killed in the nonfasting state. Harvested livers were frozen at −70° and stored at this temperature until analyzed for folate levels.

Folate analysis. Weighed samples of frozen liver were homogenized at 4° with a Brinkmann homogenizer in 4 vol (w/v) of 0.1 M sodium borate buffer, pH 9.3, containing 0.2% ascorbate and 10 mM dithioerythritol. The homogenates were immediately poured into a test tube in a boiling water bath that contained 6 additional vol. of the same borate/ascorbate/dithioerythritol buffer. After boiling for 15 min, the homogenates were cooled in an ice bath and centrifuged at 20,000 *g* for 15 min. The supernatant fractions were collected and stored at −70° in vacutainer tubes until analyzed. These extraction conditions, which differ from those we used previously [19, 26], minimized the hydrolysis of folylpolyglutamates to shorter chain derivatives during extraction (see Fig. 1). These conditions also preserve the substitutions on the pteridine ring except for 5-formyltetrahydrofolates, which isomerize to their respective 10-formyltetrahydrofolates. Thus, any 5-formyltetrahydrofolate in the liver [27, 28] is identified with 10-formyltetrahydrofolates, one disadvantage of these extraction conditions. Full details of this procedure will be described in a forthcoming publication.

A portion of each of the supernatant fractions, containing an estimated folate content not exceeding 15 nmol, was mixed with a trace amount of [^{3}H]folic acid (0.2 μCi) and applied to a folate binding protein-Sepharose affinity (1 mL bed volume) column [29]. The column was washed with 1 M potassium phosphate buffer (pH 7.0) and water and eluted with (4 × 1 mL) 0.02 M trifluoroacetic acid containing 0.01 M dithioerythritol. The acidic fractions were promptly neutralized with 1 M piperazine, and aliquots were used for tritium counting. A recovery of 90% of tritium was taken as an indication that the affinity column was not overloaded. Fractions containing the radioactivity were combined, and 1–2 mL was used for analysis of folate content and distribution, using the ion pair reverse phase high performance liquid chromatography column described in detail elsewhere [19, 26]. Briefly, the sample was injected onto a C18 HPLC column (Econosphere, 5 μm, 4.6 × 100 mm; Alltech, Deerfield, IL) equilibrated with 5% tetrabutyl ammonium phosphate, 25 mM NaCl, 5 mM dithioerythritol and 10% acetonitrile. Folates were eluted from the column using a linear gradient (10–65%) of acetonitrile in the same equilibration solution, and activity was monitored by a diode array UV detector (Hewlett Packard) that was set up to record absorption at 280, 258, and 350 nm. Folate elution by this column is in the form of clusters representing folates with increasing numbers of glutamate residues. Each cluster consists of folates that contain the same number of glutamate residues but differ in the pteridine ring substituents. In this specific study, only three forms of pteridine ring substituents were found. These included 10-formyltetrahydrofolates and unsubstituted tetrahydrofolates that eluted in the same peak within each respective cluster, and 5-methyltetrahydrofolates that eluted as a separate peak in each cluster. Quantitative resolution of peaks containing 10-formyltetrahydrofolates and unsubstituted tetrahydrofolates was made on the basis of integrated peak areas at 280 and 258 nm [19, 26]. Integrated peak areas at 280 nm were used for the estimation of 5-methyltetrahydrofolate concentrations in the respective peaks. A mixture of $PteGlu_{1-7}$ standards was also run to determine the outer boundary of each cluster, hence the glutamate content of each of the eluting folates in the sample runs.

Protein concentrations were determined by the method of Lowry *et al.* [30].

Statistical analysis. Data were analyzed for statistical significance using Student's *t*-test, and a value of $P < 0.05$ was accepted as significant. Values cited in the text represent means ± SEM.

RESULTS

The initial weights of the rats before the experimental period approximated 180 g. After 4 weeks, rats fed ethanol, unlike controls, lost weight (Table 1) but showed similar liver weights.

Figure 1 illustrates typical chromatograms from the ion pair liquid chromatography of purified folates in liver extracts of the two groups. The chromatographic pattern in the top panel of Fig. 1 is typical for liver folates from normally fed rats

Table 1. Body and liver weights of rats fed a semi-liquid diet with and without alcohol

Diet group	N	Body weights (g)	Liver weights (g)
Control	5	219.8 ± 4.2	6.04 ± 0.34
Alcohol	6	162.7 ± 5.5*	6.98 ± 0.31 (P = NS)

Values are means ± SEM.
* Significantly different from control ($P < 0.002$).

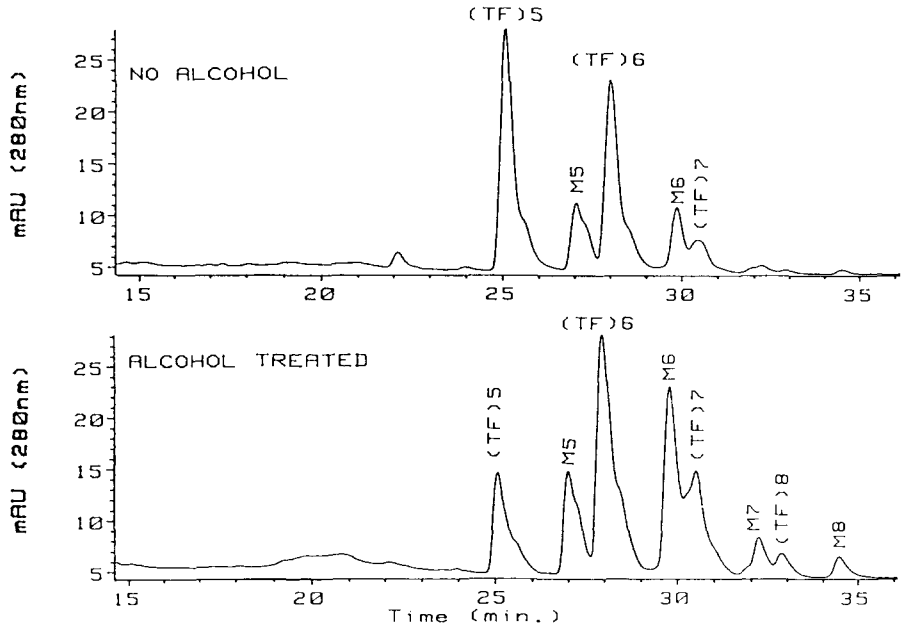

Fig. 1. Representative chromatograms of purified liver folates from two groups of rats. Top panel: liver from a control rat. Bottom panel: liver from an alcohol-fed rat. For these figures absorption at 280 nm was extracted from the continuous spectra, which were used to resolve the complex peaks, as described under Materials and Methods. Identification of the folate derivatives in the various peaks is denoted as: F, 10-formyltetrahydrofolates; T, unsubstituted tetrahydrofolates; and M, 5-methyltetrahydrofolates. Numbers represent the total number of glutamate residues.

Table 2. Effect of alcohol ingestion on the glutamate chain lengths of hepatic folates

		Glutamate chain lengths distribution (% of total folates*)		
Diet group	N	Glu5	Glu6	Glu7
Control	5	47.90 ± 4.58	46.50 ± 1.92	5.80 ± 1.91
Alcohol	6	29.16 ± 2.56†	54.92 ± 1.75†	15.18 ± 1.74†

Values are means ± SEM.
* See Table 4 for total folate values.
† Significantly different from control ($P < 0.01$).

[19, 26]. These folates are represented by two major clusters containing penta- and hexaglutamyl derivatives and a minor cluster containing hepta-glutamyl folate derivatives. In each cluster, the first peak contains 10-formyl-$H_4PteGlu_n$ (Fn) and unsubstituted $H_4PteGlu_n$ (Tn) and the second peak 5-methyl-$H_4PteGlu_n$ (Mn). As seen, the chromatographic pattern of liver folates from the alcohol-fed rats (Fig. 1, bottom panel) differed from that of controls by the occurrence of additional peaks at retention times greater than 31 min, as well as different intensities of the various peaks. The differences between the two groups, summarized for polyglutamyl chain length, are listed in Table 2. The differences, grouped by substituents on the pteridine ring, are summarized in Table 3.

The additional peaks seen in the chromatograms of liver folates from alcohol-treated rats represent poly-glutamyl folates containing more than 5 glutamate residues added to the folate molecule. When expressed as glutamate chain length relative distribution (Table 2), alcohol treatment was seen as causing a decrease in the proportion of pentaglutamyl folates together with an increase in the proportion of hexaglutamyl and heptaglutamyl derivatives.

One difference in peak intensities between the two chromatograms is in the peaks which represent methyltetrahydrofolates (denoted as Mn in Fig. 1). Table 3 shows that the total methyltetrahydrofolate concentration in livers from alcohol-fed rats was significantly higher than that from control rats. Another difference was in the liver concentrations of 10-formyltetrahydrofolates (including 5-formyl-tetrahydrofolates): those from alcohol-treated rats were significantly lower than those from control rats. Unsubstituted tetrahydrofolates were also higher in

Table 3. Effect of alcohol ingestion on the pteridine ring distribution of hepatic folates

Diet group	N	Pteridine ring distribution of hepatic folates (% of total folates*)		
		5-methylTHF	THF	10-formylTHF
Control	5	14.80 ± 1.23	38.88 ± 2.50	46.18 ± 2.65
Alcohol	6	21.46 ± 2.21†	52.91 ± 3.84‡	25.62 ± 4.02†

Values are means ± SEM.
* See Table 4 for total folate values.
† Significantly different from control ($P < 0.01$).
‡ $P = 0.082$.

Table 4. Hepatic folate concentrations in rats fed semi-liquid diet with and without alcohol

Diet group	N	Hepatic folate concentration	
		nmol/g wet weight	nmol/g protein
Control	5	25.28 ± 1.25	134.72 ± 3.30
Alcohol	6	25.90 ± 1.70 (P = NS)	126.66 ± 3.77 (P = NS)

Values are means ± SEM.

the samples from the alcohol-fed rats compared with those from control rats, but this difference did not reach statistical significance ($P = 0.082$, Table 3).

Table 4 shows that the total hepatic folate concentration was not different between the two groups whether expressed on the basis of gram wet weight or protein content of the livers.

DISCUSSION

Hillman *et al.* [9] used radioactive folate injections into rats to propose that alcohol ingestion is associated with diminished folate release into bile, and consequently trapping in the liver of both mono- and polyglutamyl folates. The data presented in this study have demonstrated that alcohol ingestion is also associated with elongation of the glutamate chains of the endogenous folate molecules. We were unable, however, to find any detectable monoglutamyl folates in the livers of either group.

In addition, the present study demonstrated considerable differences between alcohol-treated and control rats with respect to the pteridine ring distribution of hepatic folates. Compared with control rats, livers from alcohol-treated rats contained higher proportions of methylated tetrahydrofolates and decreased concentrations of formylated tetrahyrofolates, whereas unsubstituted tetrahydrofolates tended to increase in proportion. The increases in methylated tetrahydrofolate concentrations were anticipated both because of the earlier studies of Wilkinson and Shane who showed similar increases [15] and because of the reports showing that alcohol ingestion is associated with decreased methyltetrahydrofolate:homocysteine methyltransferase activity [21–24]. The observed decrease in formylated tetrahydrofolate and increase in unsubstituted tetrahydrofolate concentrations in livers of alcohol-treated rats, however, was not anticipated nor has it been reported previously. Decreased activity of methyltetrahydrofolate:homocysteine methyltransferase satisfactorily explains the accumulation of methyltetrahydrofolates, but the changed concentrations of other tetrahydrofolates cannot be ascribed to this source. Some additional interpretation is required.

One possible explanation is that the increased reducing potential [31] within the hepatocyte, due to ethanol oxidation, could favor serine synthesis at the expense of formyltetrahydrofolate synthesis. Such an explanation has been proposed [28] to account for the observation that the addition of glucose-6-phosphate to a pigeon-liver extract (thereby generating NADPH) results in the incorporation of labeled formate into serine at the expense of 5-formyltetrahydrofolate [32]. Such a mechanism would both decrease the total formylated tetrahydrofolates and increase the unsubstituted tetrahydrofolates, the latter being the second product of serine synthesis.

Another partial interpretation is that the observed increase in unsubstituted tetrahydrofolate concentrations is the result of the abundant acetaldehyde, derived from ethanol oxidation, interfering with the normal function of tetrahydrofolate coenzymes. Acetaldehyde is capable of condensation with unsubstituted tetrahydrofolate to form 5,10-methylmethylenetetrahydrofolate (5,10-ethylenetetrahydrofolate) with an association constant at 38° of 91 M^{-1} [33]. Although this association constant is 300-fold less than the formation of 5,10-methylenetetrahydrofolate, in alcoholic rats the hepatic acetaldehyde concentration can reach 250 nmol/g tissue [34], which exceeds the con-

centration of formaldehyde (tetrahydrofolate-bound and free) by 50- to 80-fold [35]. Since the methylene group of 5,10-methylenetetrahydrofolate and the ethylene group of 5,10-ethylenetetrahydrofolate readily dissociate during chromatographic procedures to generate the free aldehydes and unsubstituted tetrahydrofolate, these three folate compounds would all be identified as "unsubstituted tetrahydrofolate" by the analytical method used in this work. Thus, the apparent increment in tetrahydrofolate is an increment in the sum of these three compounds, and could include any 5,10-ethylenetetrahydrofolate resulting from the interaction of acetaldehyde and tetrahydrofolate.

Glutamate chain elongations of folates were encountered previously in states of folate deficiency caused by restriction of dietary folate supply or by chronic administration of methotrexate [26, 36, 37]. It may be that the putative interference by ethanol (or acetaldehyde) with folate-dependent one-carbon metabolism creates conditions that mimic those that are created by states of folate deficiency, hence the glutamate chain elongations. The alternate interpretation, which linked these chain elongations to alcohol-related trapping of hepatic folates because of impaired biliary folate excretion [9], is inconsistent with our data which showed that total folate concentrations in the alcohol-treated group are not higher than those in the control groups irrespective of whether these concentrations are expressed per gram wet weight or per gram protein.

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