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Effect of alcohol on tumor folate supply*

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During periods of sustained alcohol ingestion, rapidly proliferating tissues such as the erythroid marrow demonstrate a reversible defect in DNA synthesis and cell replication which appears to result from an alteration of folate homeostasis [1–4]. With acute alcohol ingestion serum folate levels fall dramatically, and megaloblastic erythropoiesis may occur even when liver folate stores are still present [4, 5]. Recovery is prompt upon withdrawal of alcohol without folate supplementation, suggesting a reversible *in vivo* sequestration of folate. Hillman *et al.* [6] have shown that levels of bile folate fall dramatically with acute alcohol ingestion and suggest a specific toxic effect of alcohol on the folate enterohepatic cycle. However, direct studies of the effects of alcohol on peripheral tissue folate supply have not been available, since normal tissues do not take up sufficient amounts of folate. In this study, we have used a new animal model where multiple subcutaneous fibrosarcoma implants permit kinetic measurements of folate transport by serial sampling of the tumor nodules [7].

Female Wistar–Furth rats weighing 150–200 g were used for all experiments. In each animal, two 2–5 g non-metastasizing fibrosarcoma tumor nodules were produced by injections of 0.5 ml of a homogenate of a tumor cell line infected with polyoma virus DW-7410 obtained from Dr. Peter Wright (Swedish Hospital Tumor Institute). Three groups of animals were compared: (1) normal animals maintained on standard Purina rat chow containing 30 μg/g of *Lactobacillus casei* active folate; (2) folate/nutrient deprived animals (F/ND) fed a liquid diet of 25% sucrose in water with 1 mg/ml of succinyl sulfathizole (added to suppress intestinal production of folates) for 3 days; and (3) alcohol-treated animals (EtOH) fed 100 ml/kg per day of a solution of 10% ethanol (in divided doses) by stomach tube for 3 days, together with the succinyl sulfathizole/sugar water diet. Animals from each of the three dietary groups were anesthetized with ether and injected with 5 μCi (100 ng) of [^{3}H]PteGlu$_1$ by tail vein. The isotope was obtained from the Amersham Corp. (Arlington Heights, IL) and had a specific activity of 20 Ci/mmole with greater than 90–95% purity when chromatographed on DEAE-A-25 Sephadex just before use [8]. No significant increase in serum folate levels was observed with this dose. After 3 hr, the animals were again anesthetized, and a tumor nodule was removed. The nodule was immediately weighed, chopped, and homogenized in a solution of cold 1% ascorbate in 0.1 M phosphate buffer, pH 6.0, boiled for 7 min to inactivate gamma-glutamyl carboxypeptidase, autoclaved at 110° for 10 min, and centrifuged to precipitate remaining protein. A 1-ml aliquot of the supernatant fraction was pipetted into Aquasol for counting. An aliquot was also used for chromatography on a 0.9 by 120 cm column of Sephadex G-15, eluted with 0.1 M potassium phosphate buffer, pH 7.0, containing 200 mM 2-mercaptoethanol [9]. The fluid was collected in 1.6-ml fractions at a flow rate of 15 ml/hr, and 1 ml of each fraction was pipetted into Aquasol for counting, PteGlu$_1$, CH_3H_4PteGlu$_1$ and polyglutamate markers were used to verify peak positions. After 6 hr, each animal was again anesthetized, the portal vein was cannulated, and the liver was flushed with cold saline. The liver and remaining tumor nodule were then removed, weighed and immediately homogenized in cold 1% ascorbate in 0.1 M phosphate buffer, pH 6.0, and then prepared for counting and chromatography as above. Serum folate levels were determined by the aseptic *L. casei* method of Herbert [10]. Counting of tissue extracts and chromatographic eluates was carried out in a Packard liquid scintillation counter, and correction for quenching was made using the external automatic standard. The amount of polyglutamyl folate present was calculated as the percentage of total labeled folate in the tissue. Statistical analysis was performed by Student's *t*-test.

The serum folate levels of EtOH animals (41 ± 3 ng/ml) were significantly lower than in F/ND animals (63 ± 8 ng/ml; $P < 0.05$) which were, in turn, lower than normals (92 ± 9 ng/ml; $P < 0.02$). The uptakes of labeled folate per g of tumor tissue for the three groups are shown in Table 1. After 3 hr, tumor uptake in EtOH animals was only slightly less than that observed in F/ND animals but significantly less than in normals ($P < 0.05$). By 6 hr, tumor uptake in EtOH animals was significantly less than that seen in both normal and F/ND animals ($P < 0.05$ and $P < 0.05$ respectively). When hepatic uptakes were examined at 6 hr, F/ND animals appeared to take up more label than either normal ($P < 0.01$) or EtOH animals ($P < 0.02$).

Chromatographic analysis of labeled folate taken up by tumor tissue (Table 2) demonstrated that significantly more

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Table 1. Tissue uptake of [^{3}H]PteGlu$_1$*

		[^{3}H]PteGlu$_1$ uptake (% of injected dose 1 g wet wt)		
		Normal (N = 10)	F/ND (N = 7)	EtOH (N = 5)
Tumor	3 hr	0.55 ± 0.07	0.44 ± 0.05	0.37 ± 0.03
	6 hr	0.8 ± 0.07	0.79 ± 0.10	0.51 ± 0.09
Liver†	6 hr	1.57 ± 0.15	2.36 ± 0.24	1.56 ± 0.20

* Results are means ± S.E.M. for number of animals shown.

† The *total* liver uptake (percent of injected dose, mean ± S.E.M. for number of animals shown) was: normal = 10.5 ± 1.0; F/ND = 14.0 ± 1.5; and EtOH = 11.3 ± 2.2.

Table 2. Conversion of labeled folate to polyglutamyl folate at 6 hr*

	% Total tissue activity				
	Tumor			Liver	
Normal	41.0 ± 1.8	$P < 0.07$	$P < 0.002$	50.7 ± 5.3	$P < 0.005$
F/ND	30.5 ± 2.9	$P < 0.001$		28.2 ± 1.7	$P < 0.001$
EtOH	52.4 ± 3.0			44.4 ± 5.0	

* Each result is the mean ± S.E.M. for five animals.

labeled folate was converted to polyglutamyl folate by 6 hr in EtOH animals than in normals, which in turn was greater than that in F/ND animals. Studies of hepatic polyglutamyl folate formation at 6 hr showed less labeled polyglutamyl folate in F/ND animals than either EtOH ($P < 0.03$) or normal animals ($P < 0.01$).

Studies of alcohol toxicity in man and animal models have demonstrated that acute alcohol ingestion results in a dramatic fall in serum folate levels which is not matched by a similar reduction in hepatic folate [4–6, 11]. This contrasts with the response to folate deprivation in which intrahepatic folates are mobilized as the serum folate level falls [11]. The difference may be the result of a toxic effect of alcohol on hepatocyte folate metabolism and the folate enterohepatic cycle [6, 12, 13]. Alcohol appears to decrease the transport of folate from the hepatocyte to bile, diverting it instead to an intracellular polyglutamyl folate pool. This could explain the alcohol-related fall in the serum folate level, since studies of normal folate kinetics have shown that the serum folate depends, in part, on folate circulation through the enterohepatic cycle [8].

The present study extends this work by demonstrating that folate supply to peripheral tissue is also compromised by acute alcohol ingestion. While this might be predicted from the clinical observation that alcohol can rapidly induce megaloblastic hematopoiesis [3, 4], this work provides the first direct demonstration of decreased supply to tissue. Coupled with the recent evidence of the important contribution of the folate enterohepatic cycle to the supply of folate to subcutaneous fibrosarcoma nodules [7], these results support the postulate that alcohol interference with transport of folate from the hepatocyte to bile not only causes a fall in the serum folate but also may reduce folate transport to tissues.

This work also provides additional data on the effect of alcohol on polyglutamyl folate pools in liver and tumor. Although a preliminary uncontrolled report suggested that alcohol may decrease hepatic polyglutamyl folate accumulation [14], this work supports previous studies by Hillman *et al.* [6] which demonstrated an increase in intracellular polyglutamyl folates in the face of acute alcohol ingestion. The tumor also demonstrated an increased accumulation of label as polyglutamyl folate when compared to either normal or F/ND animals (Table 2). Thus, alcohol-induced accumulation of folate in the intracellular polyglutamyl folate pool is not limited to liver. Furthermore, this appears to be a direct effect of alcohol ingestion either by stimulating polyglutamyl folate sytnhesis or inhibiting loss or breakdown, rather than a response to folate deficiency, since it was not observed in F/ND animals.

In summary, the results show that the known toxic effect of alcohol on the enterohepatic cycle is associated with a major reduction in folate supply to a peripheral tissue (in this case, tumor) together with an increased accumulation of tumor polyglutamyl folate similar to that reported for the hepatocyte.

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1,1-Dichloroethylene inhibition of liver endoplasmic reticulum calcium pump function

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1,1-Dichloroethylene (vinylidene chloride, 1,1-DCE) is a potent hepatotoxin that, in contrast to agents such as CCl_4, does not produce lipid peroxidation in liver membranes [1, 2]. Electron microscopic examination of livers from 1,1-DCE-treated, mature animals clearly demonstrates damage at the nucleus, plasma membrane and mitochondria [3, 4]. Again, in contrast to agents such as CCl_4, liver endoplasmic reticulum (ER) damage does not appear as an early morphological change after 1,1-DCE administration to mature rats [4]. Significant biochemical changes of ER function are thought to occur late in 1,1-DCE toxicity. Inhibition of the mixed function oxidase system (MFOS) and of glucose-6-phosphatase (G6Pase) in ER is not observed until 1,1-DCE-induced liver damage is well established [4]. However, a recent report shows that activity of an ER calcium transport pump is dramatically decreased 2 hr after 1,1-DCE administration [5]. The current study characterizes the time course of 1,1-DCE effect on a calcium transport system in liver ER and on ER calcium levels at times up to 4 hr after 1,1-DCE administration. The results demonstrate that dramatic changes of ER function occur very early after 1,1-DCE administration. This observation may suggest that, in common with CCl_4 and other hepatotoxins, ER damage occurs early in the sequence of 1,1-DCE intoxication. This observation lends additional support to the hypothesis that alteration of intracellular calcium may initiate a series of events that produce cell death [5–8].

Male Sprague–Dawley rats (250–400 g) were used for this study. Animals were allowed free access to food and water throughout the experiments. 1,1-DCE (Aldrich Chemical Co., Milwaukee, WI) was diluted in corn oil and administered i.p. In some experiments, rats were pretreated with diethylmaleate (DEM, Sigma Chemical Co., St. Louis, MO) (0.6 ml/kg i.p.) 1 hr before 1,1-DCE. In other experiments, rats were pretreated with three doses of phenobarbital (J. T. Baker Chemical Co., Phillipsburg, NJ) (80 mg/kg) 72, 48 and 24 hr before 1,1-DCE. Calcium pump activity was determined in either a microsomal fraction [5, 6] or a 12,500 *g* supernatant fraction. All calcium uptake activity in the 12,500 *g* supernatant fraction could be attributed to the calcium pump in the microsomal fraction. Calcium pump activity was measured in the following medium: 100 mM KCl, 30 mM imidazole–histidine buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP (pH adjusted to 6.8 with imidazole), 5 mM ammonium oxalate, 5 mM sodium azide, 20 μM $CaCl_2$ ([$^{45}Ca^{2+}$], 0.2 μCi/ml) and 20–50 μg microsomal protein (or equivalent 12,500 *g* supernatant fraction)/ml. The assay was initiated by addition of the membrane fraction to prewarmed assay medium (37°). At timed intervals, samples were removed, filtered through 0.45 μm nitrocellulose cellulose filters, and [$^{45}Ca^{2+}$] was determined by liquid scintillation spectrophotometry. Liver and microsomal calcium was determined by atomic absorption spectrophotometry, as previously described [5]. Glucose-6-phosphotase activity was determined as described by Aronson and Touster [9]. Glutathione was determined as described by Jaeger *et al.* [10]. Lipids were extracted from the 12,500 *g* supernatant fraction after *in vivo* administration of 1,1-DCE, and lipid peroxidation was determined as conjugated dienes at 243 nm as described by Klaassen and Plaa [11]. Protein was determined by the Lowry method as described by Shatkin [12].

As demonstrated in Fig. 1, calcium pump activity was inhibited 45% within 20 min after 1,1-DCE administration. Pump activity continued to decline and was maximally (70%) inhibited by 4 hr. In a previous study, this nadir was reached by 2 hr [5]. 1,1-DCE administration depletes GSH [4, 10] and GSH depletion potentiates 1,1-DCE hepatotoxicity [10]. GSH is important in 1,1-DCE metabolism and probably serves as a detoxification route for a reactive 1,1-DCE metabolite [13–15]. Inhibition of the calcium pump paralleled loss of GSH (Fig. 1). However, GSH depletion in itself was not sufficient to produce calcium pump inhibition. Doses of DEM of up to 2.4 ml/kg did not result in calcium pump inhibition or release of microsomal calcium when animals were killed 1 hr after DEM administration. Other evidence of 1,1-DCE-induced, ER dysfunction is presented in Fig. 1. Calcium associated with the microsomal fraction began to decline within 20 min after 1,1-DCE administration and fell to 50% of control at 4 hr. This is similar to an effect produced by CCl_4 or $CHCl_3$ [5]. In contrast to these effects on ER calcium systems and in agreement with previous findings, 1,1-DCE did not alter microsomal G6Pase activity [5] or the level of conjugated dienes extracted from microsomes [2, 5] (Fig. 1).

Evidence of extensive liver damage was obvious at 4 hr. At this time, serum glutamic pyruvic transaminase activity