the 10-min desensitizing exposure to CD no reduction in mechanical response was seen. Furthermore, no desensitization is seen after K^+ exposure (K. Jim and D. J. Triggle, unpublished results) although K^+ and CD responses are equally sensitive to Ca^{2+} channel antagonists [11]. More plausibly, the hyperpolarization mediated by electrogenic Na^+ pumping following removal of a muscarinic agonist in the ileal longitudinal muscle [17, 18] reduces the activity of depolarizing agonists [19]. This suggestion is consistent with our previously reported suggestion that A23187 action in this tissue involves a Na^+-dependent depolarization mediating activation of voltage sensitive Ca^{2+} channels [6]. In any event, the densensitization of A23187 responses by prior muscarinic receptor activation is not consistent with A23187 serving as a simple Ca^{2+} carrier.

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Effect of alcohol on hepatic secretion of methylfolate ($CH_3H_4PteGlu_1$) into bile*

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Acute alcohol ingestion is associated with a rapid fall in serum folate level that, in part, may relate to an alcohol effect on the folate enterohepatic cycle. Past experiments with an alcoholic rat model showed a marked decrease in biliary folate matched by increased formation of hepatic polyglutamate [1]. Previously, Brown *et al.* [2] had reported a reduction in liver polyglutamate formation in alcoholic rat liver. Lane *et al.* [3] postulated a blocking effect of alcohol on mobilization and transport of folate from tissue to plasma in alcoholic man. The latter study employed the flushing technique of Johns *et al.* [4] to measure the rate of tissue storage and utilization of $^{14}CH_3H_4PteGlu_1$.

To further investigate the different results obtained in these studies, we examined the effect of the flushing technique on release of isotopic folate from liver in the alcoholic rat model. The tendency for an increased conversion to polyglutamate was again demonstrated. However, a simultaneous resistance to flush of labeled folate from the alcoholic hepatocyte was also apparent, suggesting an alcohol-induced defect in the transport of folate into bile.

Female Sprague–Dawley rats weighing 150–250 g were used. Three groups of animals were compared: (1) normal animals maintained on a standard Purina rat chow diet containing 30 μg/g of *Lactobacillus casei* active folate; (2) folate/nutrient deprived (F/ND) animals maintained by feeding tube for 3 days on a liquid diet of 25% sucrose in water to which 100 mg of succinyl sulfathiazol/100 ml was added to suppress intestinal production of folate; and (3) folate/nutrient deprived alcoholic animals (F/ND–EtOH) maintained by feeding tube on 100 ml/kg per day of a solution of 10% ethanol for 3 days, together with the succinyl sulfathiazol/sugar water diet. After 3 days, bile duct cannulation was performed as described previously [1]. While still under anesthesia, 100 ng of [^{3}H]$PteGlu_1$ (sp. act. 20 Ci/mmole) was injected by tail vein. All bile was then collected for the next hour, including 20 μl samples at 10-min intervals for isotopic counting. At the end of 1 hr, five control animals from each dietary group were killed; the livers were removed, weighed, and immediately prepared for counting and chromatography [5]. An additional five control animals from each group were killed after 2 hr for chromatographic analysis of liver folate.

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Three animals from the normal diet and F/ND groups and six from the F/ND–EtOH group were used for the flush experiment. At the end of the first hour of bile drainage, a 1 mg dose of unlabeled $PteGlu_1$ was administered by tail vein followed by 0.17 mg every 10 min over the next hour. Both bile and urine were collected throughout the second hour, followed by killing of the animals and recovery of the liver for counting and chromatography. Twenty μl samples of bile were again collected at 10-min intervals during the second hour for counting.

Chromatography of liver extracts, bile, and urine was carried out on 0.9 × 100 cm columns of Sephadex G15-120. The effluent was collected at 1.6-ml fractions and pipetted into Aquasol for counting. To identify $CH_3H_4PteGlu_1$, an aliquot of eluate volumes 125–150 ml was placed on a 1.5 × 30 cm column of Sephadex DEAE 25 [5]. Total labeled folate in bile and liver was expressed as a percentage of total label injected. The ratio of mono- to polyglutamate in liver was determined chromatographically. Serum folate levels were determined by the aseptic *L. casei* assay [6]. Data are expressed as means ± S.E.M. and statistics performed by the Student's *t*-test.

At the time of study, serum folate levels of F/ND–EtOH animals (43 ± 13 ng/ml) were significantly lower than either normal (92 ± 15 ng/ml, $P < 0.05$) or F/ND animals (75 ± 9 ng/ml, $P < 0.01$). The appearance of labeled folate in bile 1 hr after the intravenous injection of $[^3H]PteGlu_1$ was decreased in both F/ND ($P < 0.005$) and F/ND–EtOH ($P < 0.004$) compared to normal (Fig. 1 and Table 1). With the flush dose of $PteGlu_1$, all groups demonstrated a rapid increase in the output of labeled $CH_3H_4PteGlu_1$ into bile, though there was considerable individual animal variation (Fig. 1). The mean recovery of isotope in bile during the second hour post flush was the same for F/ND and F/ND–EtOH animals. However, both were significantly below that observed for the normal diet animals ($P < 0.015$ for F/ND and $P < 0.04$ for F/ND–EtOH).

A difference between F/ND and F/ND–EtOH animals was apparent when the liver was studied. As shown in Table 1, the percentage of isotope remaining in liver after flush was significantly greater for alcoholic animals ($P < 0.026$) when compared to F/ND animals. In fact, the amount of isotope remaining in the livers of F/ND–EtOH animals was even greater than that seen in normal animals ($P < 0.03$). Moreover, this was associated with a change in the ratio of labeled mono- (Glu_1) to polyglutamate (Glu_5). The presence of alcohol prevented the normal response to folate deprivation, that is, a shift of the ratio toward the monoglutamate form.

Past studies have suggested several toxic effects of alcohol on the hepatic handling of folate. Prolonged alcohol ingestion with liver damage may be associated with decreased folate uptake and polyglutamate formation [2]. Acute alcohol ingestion has been reported to cause increased hepatocyte retention, with diversion, of folate into the intracellular polyglutamate pool at the cost of transport into bile [1, 7]. The latter studies were carried out in an acute alcoholic rat model that combined gastric tube feedings of ethanol with folate and nutrient deprivation. When the handling of $[^3H]PteGlu_1$ was studied over a 6-hr period, it was apparent that, despite normal hepatic uptake, labeled folate secretion into bile was reduced while polyglutamate formation was increased. The underlying mechanism of this effect, whether interference with transport from hepatocyte to bile or stimulation of a normal or abnormal polyglutamate formation, was not defined.

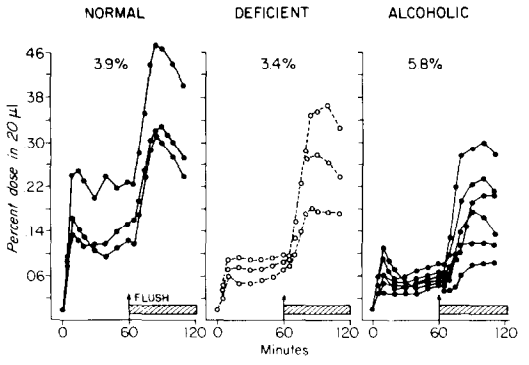

Fig. 1. Folate secretion in bile. The labeled folate secretion in bile over time (as the percentage of administered dose/20 μl sample) is shown for three normal, three deficient and six alcoholic animals. Beginning at 60 min, flushing doses of $PteGlu_1$ were administered (hatched area). The percentage of the administered dose remaining in the liver at 120 min is shown (mean for each group).

In an attempt to distinguish between these two effects, the ability to mobilize labeled folates from the liver with the flushing technique of Johns *et al.* [4] was studied in the same acute alcoholic rat model. Both normal and folate/nutrient deprived animals were simultaneously studied as controls. The results confirmed the previously reported effect of alcohol on the uptake, storage, and release of folate by the liver *in vivo*. Alcohol appeared to counteract the normal response of the animal to folate/nutrient deprivation, that is, a shift in the intracellular mono- to polyglutamate ratio toward the monoglutamate form. Instead, the alcoholic liver continued to produce polyglutamate in a ratio similar to that for normal liver despite a major fall in both biliary and serum folate levels. At the same time, the results also suggested that the ability to flush folate monoglutamate from alcoholic rat livers is abnormal. The amount of labeled folate retained by the alcoholic livers was significantly greater than normal and folate/nutrient deprived. Although these findings do not resolve the issue completely, they are compatible with a toxic effect of alcohol directed at the transport of folate from the hepatocyte into bile. This could involve membrane transport *per se* or the intracellular binding of folate monoglutamate.

Table 1. Percent distribution of radioactivity in bile and liver following injection $[^3H]Pte\ Glu_1$ (100 ng, sp. act. 20–40 Ci/mmole)*

	Bile		Liver		
	1 Hr	Post flush	1 Hr	Post flush	Glu_1Glu_5 ratio
Normal	6.5 ± 1.6	14.7 ± 2.8	8.6 ± 1.4	4.1 ± 0.4	2.2/1
F/ND	2.8 ± 0.6	8.5 ± 1.6	11.4 ± 2.9	3.2 ± 1.2	4.5/1
F/ND–EtOH	3.1 ± 1.1	8.1 ± 2.9	9.4 ± 1.0	5.8 ± 1.8	1.8/1

* Values are means ± S.E.M. For the studies of distribution in bile, N = 10; for liver, N = 5.

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Liver fructose bisphosphatase concentration and activity in alloxan induced diabetes

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In a previous publication, we reported on the development and application of a radioimmunoassay for rabbit liver and kidney fructose bisphosphatase (EC 3.1.3.11) [1]. A 2- to 3-fold increase in concentration was observed following the onset of alloxan diabetes. A proportional increase in activity also occurred so that the specific activity remained unchanged in the untreated, acutely diabetic rabbit. In the present study, we elaborate on the influence on this enzyme of both untreated and insulin-treated diabetes. The application of a specific radioimmunoassay allowed us to measure the concentration of the enzyme independently of its activity.

Forty-five New Zealand rabbits weighing approximately 1200 g each and of both sexes were used in this study. All rabbits were maintained on a conventional, commercial rabbit chow diet until they were killed. Diabetes was induced by a single intravenous injection of alloxan (100 mg/kg) administered under ketamine hydrochloride and promazine anesthesia. Diabetes was established, usually at 72 hr, by the finding of a blood glucose value of approximately 500 mg/dl or of massive glucosuria. Such animals, if left untreated beyond 72 hr, died within the next few days. Lente insulin (U-40) was obtained from the Eli Lilly Co., Chicago, IL. Preparation and evaluation of goat gamma-globulin against rabbit liver frutose bisphosphatase and the maximum activity assay at 24° have been described [1]. Radioiodination of fructose bisphosphatase was accomplished using a modification of the method of Bolton and Hunter [2]. The ester was iodinated to a specific activity of about 5 mCi/μg. The enzyme was labeled to the extent of approximately 12 ^{125}I atoms/tetramer. Radioimmunoassay of fructose bisphosphatase was done as described [1] and utilized rabbit anti-goat IgG gamma-globulin as the second stage precipitation system. Animals were anesthetized and exsanguinated and the livers were removed and weighed. Homogenates (20%, w/v) were prepared and centrifuged at 115,000 *g* for 60 min. For activity measurements 10 μl of each supernatant fraction was assayed; the assay mixture contained an AMP-removal system [1]. It was established for even the most active extracts that 10 μl never exceeded the capacity of the assay to measure V_{max}. For enzyme concentration measurements, 1:10,000, 1:1,000 and 1:100 dilutions of the 115,000 *g* cytosol preparation were made with 1% egg albumin buffer. Twenty and 50 μl aliquots of each dilution were analyzed by radioimmunoassay.

Homogeneous enzyme for iodination and for use as a standard in the radioimmunoassay procedure was prepared from frozen livers of young animals, as described by Ulm *et al.* [3]. The enzyme had a specific activity of 17 units/mg at 24° (2.5 units/nmole). When the purified enzyme was maintained at 100° in 8 M urea and 1% dodecylsulfate for 5 min and submitted to SDS gel electrophoresis, even 48 μg gave only one visibly stained band. Standard enzyme was assayed for concentration by a fluorescamine assay [4, 5]. Enzyme activity was localized in 3.65% polyacrylamide slab gels utilizing principles presented by Gabriel [6]. An overlay of 1% agar containing the fructose bisphosphatase assay mixture [2], meldola blue dye (2.5 mg/100 ml), and nitrotetrazolium salts (25 mg/100 ml) was prepared and allowed to develop in the dark. Control gels did not contain fructose bisphosphatase. Data are presented as the mean ± one standard deviation.

The specific activity of the purified enzyme (2.5 units/nmole) measured by the Lowry method for protein was almost the same as that of liver extracts from normally fed rabbits (2.6; see Table 1) measured by our radioimmunoassay. The excellent agreement argues against potential problems with the radioimmunoassay, such as interference by non-specific proteins.

The concentration of fructose bisphosphatase and the units of activity per gram of liver were above normal in the diabetic animals (Table 1, No. 2), which caused a net increase of 144 nmoles of enzyme (157 to 301) per liver and of 351 units of activity per liver (413 to 764). No change in specific activity occurred, because mass and activity both changed 2-fold. Neither variable returned to normal after treatment with insulin. Even after 10 days of daily insulin injection (Table 1, No. 6), enzyme content and activity continued to increase. Enzyme content was 9-fold greater than control values, but activity was only 4-fold greater. This resulted in a decrease in specific activity to 1.3 units/nmole. The situation was essentially the same after 15 days of insulin replacement therapy (Table 1, No. 7). During the time between alloxan administration and sacrifice, animals of groups 5, 6 and 7 had total body weight