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Effects of methionine deficiency and ethanol ingestion on acetaminophen metabolism in mice

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**EFFECTS OF METHIONINE DEFICIENCY AND ETHANOL INGESTION ON
ACETAMINOPHEN METABOLISM IN MICE**

Iowa State University

Ph.D. 1985

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Effects of methionine deficiency and ethanol ingestion
on acetaminophen metabolism in mice

by

Marla M. Reicks

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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GENERAL INTRODUCTION

Acetaminophen has been available as an over-the-counter analgesic and antipyretic in the United States since 1955. In addition to its role in headache, cough and cold relief, studies have substantiated the efficacy of acetaminophen (ACAP) in the treatment of pain associated with episiotomy, osteoarthritis and dentistry (1). The best known preparation of ACAP sold in the United States is Tylenol. In 1982, this product accounted for 35% of all over-the-counter analgesics marketed (2).

When used in recommended dosages, ACAP is considered to be safe and effective (3). ACAP is metabolized in the liver primarily as glucuronide or sulfate conjugates. A small proportion of ACAP is oxidized via the microsomal cytochrome P-450 mixed function oxidase system, producing a toxic electrophilic metabolite, that can be inactivated by glutathione conjugation. If ACAP is taken in excessive dosages, a much larger proportion is metabolized to the toxic metabolite as the normal glucuronidation and sulfation pathways are overwhelmed. As glutathione is depleted by conjugation of the metabolite, more of the toxic metabolite becomes available for binding to hepatic macromolecules and thereby produces cellular necrosis.

The potential for ACAP-induced liver necrosis, therefore, depends on several factors. These include the total quantity of ACAP ingested, the plasma levels of ACAP, the rate of elimination, the relative activity of the metabolic pathways of glucuronidation, sulfation,

oxidation by the cytochrome P-450 system, and the hepatic reduced glutathione (GSH) levels. The GSH level, rather than amount of enzyme, is usually the rate-limiting factor in GSH conjugations (4).

The relative activity of the glucuronidation and sulfation may depend on the activity of the sulfotransferase and UDP-glucuronosyl transferase, and the availability of the cofactors, inorganic sulfate and UDP-glucuronic acid. The activities of the cytochrome P-450 mixed function oxidase system have been shown to be stimulated by microsomal enzyme inducers, including chronic ethanol ingestion and anticonvulsants. Increased ACAP-induced liver damage has been associated with chronic ethanol ingestion in laboratory animals and humans (5-11). As the activity of the mixed function oxidase system is increased, there may be greater production of the ACAP toxic metabolite and therefore greater glutathione depletion and increased hepatotoxicity.

Studies in animals have shown that nutritional status can directly affect hepatic glutathione levels. The extent to which cysteine is available for incorporation into glutathione depends, in part, on the activity of the hepatic transsulfuration pathway by which the methionine sulfur can be donated to the carbon skeleton of serine to produce cysteine. The activity of this pathway is dependent on the supply of methionine and the requirement of methionine for protein synthesis and methylation by S-adenosylmethionine reactions. Diets deficient in methionine have been shown to decrease hepatic glutathione levels in rats (12,13).

The purpose of this study is to determine how dietary methionine deficiency and chronic ethanol consumption affect the metabolism of ACAP in mice. This will be achieved by determining how methionine inadequacy and ethanol ingestion influence the factors that are responsible for altering the potential of ACAP to cause liver necrosis.

Explanation of Dissertation Format

This manuscript has been arranged in an alternate dissertation format. It includes this general introduction, a general review of the literature, two papers for publication and a final summary and discussion. The first section has been published in Drug-Nutrient Interactions, Volume 3, pages 43-51, 1984. The data in Section I were generated after modification of procedures used in a preceding master's thesis.

GENERAL REVIEW OF LITERATURE

Acetaminophen Metabolism

Acetaminophen (N-acetyl-p-aminophenol, paracetamol), is a commonly used non-narcotic analgesic and antipyretic. It is rapidly absorbed from the gastrointestinal tract, reaching peak plasma concentrations within 30-90 minutes and having a half-life of 2-3 hours. ACAP is relatively lipid soluble and is distributed throughout most body fluids ($V_d = 0.70 - 0.75 \text{ l/Kg}$). In humans, a clinically insignificant proportion is bound to plasma proteins. The pharmacology and toxicity of ACAP have been thoroughly reviewed recently (1-3).

ACAP is metabolized primarily in the liver (Figure 1). In therapeutic doses, the major pathways involve sulfation and glucuronidation with subsequent urinary excretion of the polar metabolites. A minor metabolic pathway for ACAP, the cytochrome P-450 mixed function oxidase system, converts a relatively small proportion of this compound to a toxic metabolite. The toxic metabolite is capable of covalently binding to hepatic macromolecules and this is thought to cause hepatic necrosis unless it is conjugated by the tripeptide, glutathione (GSH). When taken in therapeutic doses, ACAP is thought to be one of the safest of all minor analgesics. In higher than recommended doses it can cause liver necrosis. As the normal glucuronidation and sulfation pathways are overwhelmed by excessive doses, more of the reactive toxic metabolite is present for GSH

conjugation and there is increased urinary cysteine and mercapturic acid excretion. As the demand for GSH exceeds the supply, the free toxic metabolite causes hepatotoxicity, apparently by covalently binding to macromolecules.

The reactive metabolite derived from the cytochrome P-450 mixed function oxidase system activation of ACAP is believed to be N-acetyl-p-benzoquinoneimine (NAPQI) (14). Several mechanisms for the formation of NAPQI have been proposed. Initially, it was thought that ACAP was N-hydroxylated to form N-hydroxy-ACAP, followed by spontaneous dehydration to NAPQI. However, the dehydration rate seems to be too slow to support this proposal (15). Several alternate mechanisms of ACAP activation leading to NAPQI formation have been proposed which do not involve N-hydroxy-ACAP (16,17).

Although the identity of the toxic metabolite is generally believed to be NAPQI, the molecular basis for its cytotoxicity remains controversial. Holme et al. (18) incubated isolated rat hepatocytes with various concentrations of synthesized NAPQI to determine its cytotoxic effects. Cytotoxicity, as assessed by trypan blue exclusion and measurement of lactate dehydrogenase release into the medium, was detected 10-120 minutes after the incubation period began, with the longest time corresponding to the lowest NAPQI concentration. However, the half-life of NAPQI is so short, that less than 0.5% of the initial amount added could be expected to remain in the incubation medium after 2 minutes. These authors then concluded that subsequent cellular steps

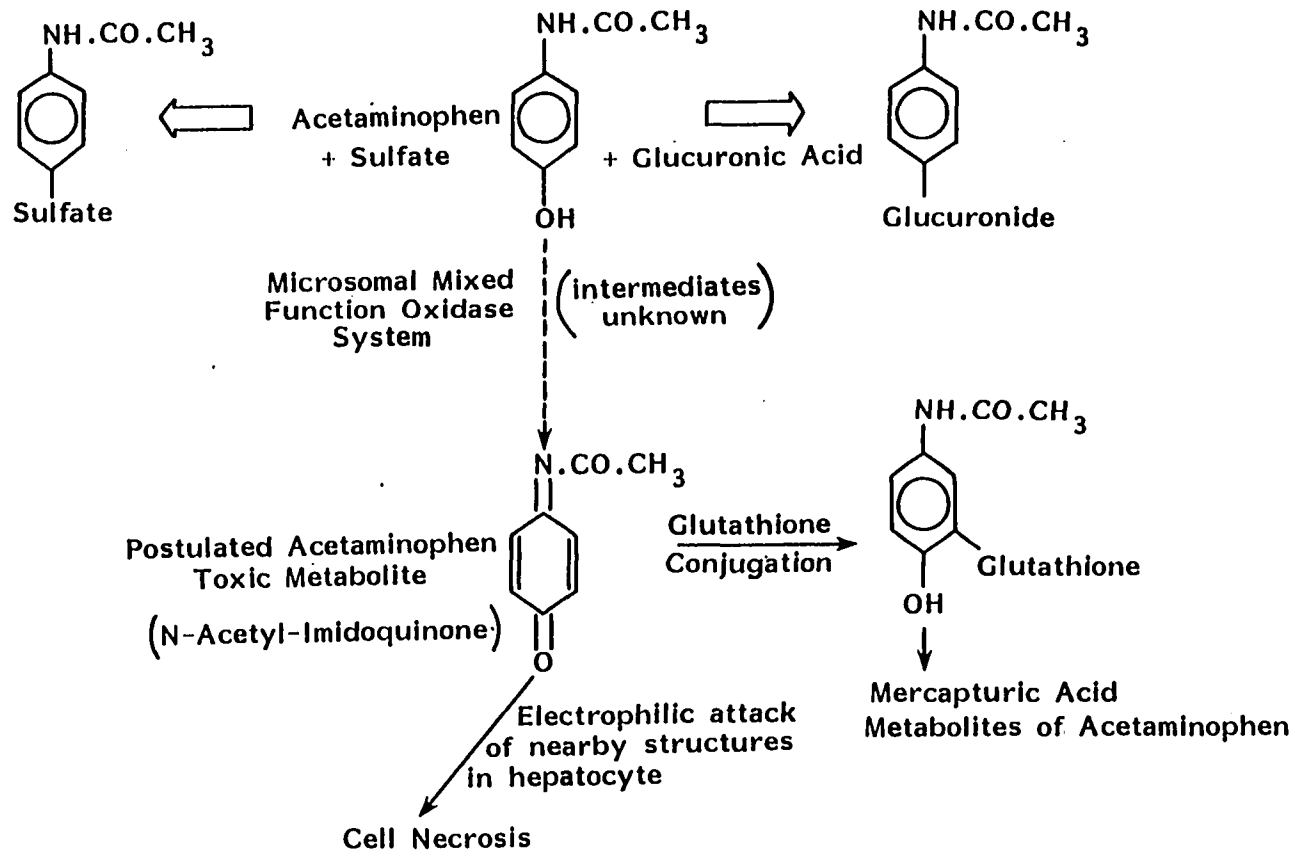


FIGURE 1. Acetaminophen metabolism

must occur after the initial damage was caused by NAPQI before cytotoxicity could be detected.

Support for the concept that the generalized covalent binding of the toxic metabolite to essential cellular macromolecules as the cause of cell necrosis has been provided by studies which correlate the extent of covalent binding with the degree of liver necrosis (19). However, further observations have disputed the validity of this concept. Devalia and McLean (20) incubated isolated rat hepatocytes with [^{14}C]ACAP and then determined the amount of covalent binding in damaged and undamaged cells. After 4 hours incubation time, the undamaged cells contained more bound ACAP than the damaged cells contained after 1 hour. After 22 hours in tissue culture incubation, the undamaged cells contained as much bound ACAP as the 1 hour killed cells. The authors suggest that covalent binding is not the main cause of ACAP induced hepatotoxicity, but only an initiator of a multi-step process leading to cell death.

Genotoxicity, measured as incorporation of [^3H]thymidine into hepatocyte DNA, was not increased after ACAP incubation in rat primary hepatocyte cultures (21), although significant cytotoxicity was observed. The toxic metabolite of ACAP seems to interact preferentially with proteins rather than deoxyribonucleic acid moieties.

It has been proposed that the ACAP toxic metabolite is preferentially bound by particular proteins critical to the cell's survival. Streeter et al. (22) presented evidence for cysteine residues

as the major sites of covalent binding of ACAP in phenobarbital pretreated mice hepatic microsomal preparations. The ACAP toxic metabolite has been shown to bind covalently to GSH S-transferases if GSH content is depleted (23). Wendel and Cikryt (24) reported that 65% of the radioactivity after [¹⁴C]ACAP was incubated with mouse liver homogenate, was bound to soluble protein. This radioactivity peak co-chromatographed with GSH S-transferase activity using molecular sieve chromatography. This evidence suggests that the toxic metabolite is bound to the GSH S-transferase. Hirayama et al. (25) also provided evidence that GSH S-transferases are the target for binding by the ACAP toxic metabolite, followed by mitochondria and plasma membrane proteins.

It has also been suggested that lipid peroxidation plays an early or major role in ACAP-induced hepatotoxicity. The occurrence of lipid peroxidation during the course of ACAP-induced cellular necrosis has been demonstrated (18,26-28). Malondialdehyde (MDA) production was observed to be dependent on GSH depletion by ACAP in mouse and rat liver preparations. Antioxidants, such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) and calcium EDTA, protected mice (29) and rat (30) liver from ACAP induced injury. However, the incidence and extent of lipid peroxidation differs after treatment with carbon tetrachloride and ACAP (18). Recently, Fariss et al. (31) observed that there is a requirement for α -tocopherol in the maintenance of hepatocyte viability during treatment with toxic chemicals. It seemed that chemically-induced cell death was dependent on cellular α -tocopherol rather than on the presence of

extracellular calcium as expected. They suggested that the depletion of α -tocopherol and GSH may be responsible for increased susceptibility to chemically-induced cell death. The significance of lipid peroxidation and cell membrane damage in the hepatotoxicity caused by ACAP remains to be determined.

In subtoxic doses, a smaller fraction of ACAP is converted to the potentially toxic metabolite, and the sulfation and glucuronidation pathways predominate. Sulfate conjugation of hydroxyl groups, either aliphatic, phenolic or more complex, require a continuous supply of inorganic sulfate to form the cosubstrate for conjugation, 3'-phosphoadenosine- 5'-phosphosulfate (PAPS). The formation of sulfate conjugates from PAPS and phenolic compounds, such as ACAP, require catalysis by an arylsulfotransferase (E.C. 2.8.2.1.). Properties of the multiple forms of the arylsulfotransferases (phenol sulfotransferases of PST) are reviewed by Jacoby et al. (32). The control of the level and distribution of PST is not well understood. Mizuma et al. (33) studied the effects of pH, thermal treatment and the simultaneous treatment with p-nitrophenol on the function of PST multiple forms for ACAP sulfation in rat liver cytosol fraction. The sulfation rates increased with pH and the thermolability of the PST increased with decreasing ACAP concentration. They concluded that sulfation at a 1 μ M ACAP concentration is mainly catalyzed by a thermolabile PST isozyme with a lower K_m for ACAP, and sulfation at the higher ACAP concentration is catalyzed by a thermostable PST isozyme with a higher K_m for ACAP.

The properties of uridine diphospho- (UDP) glucuronosyl transferase have been reviewed by Kasper and Henton (34). Glucuronidation of ACAP involves the transferase and the cosubstrate, UDP-glucuronic acid (UDPGA).

The regulation and induction of UDP-glucuronosyl transferase activity toward ACAP was studied by Hazelton and Klaassen (35). Pretreatment with 3-methylcholanthrene (3MC) or phenobarbital did not affect mouse liver UDP-glucuronosyl transferase activity toward ACAP. The conclusion that was drawn from these results was that the UDP-glucuronosyl transferase activity toward ACAP could not be categorized as the 3MC or phenobarbital inducible forms.

The capacity for glucuronidation has been found to be dependent on the concentration of the cosubstrate, UDPGA. The depletion of UDPGA by ACAP and the time required for recovery of UDPGA levels in rat liver were found to be dependent on ACAP dose (36). The amount of UDPGA required for conjugation of a therapeutic dose of ACAP was found to be almost equal to the total amount in the liver at the normal concentration of UDPGA. The capacity to synthesize UDPGA via the glucuronic acid pathway may be the limiting factor for glucuronidation of ACAP.

Factors that determine the rate of sulfate or glucuronate conjugation include the rate at which ACAP becomes available for conjugation, the availability of the cosubstrate for conjugation, and the competition of sulfate and glucuronate for ACAP in the same cell.

At low doses of ACAP administration to the rat, sulfation has been shown to be the primary metabolic route of excretion and at higher doses, there is a shift to glucuronidation (37).

In five healthy male human subjects, Clements et al. (38) found that the percentage of an intravenous ACAP dose excreted as the urinary sulfate conjugate was higher after a 5 mg/kg dose than after a 20 mg/Kg dose. Also, the total plasma and renal clearance of the sulfate conjugate was higher after the lower ACAP dose. Morris and Levy (39) found that 2 hours after administration of 1.5 g ACAP to adult human subjects, the renal clearance ratios (relative to creatinine) was highest for ACAP sulfate and was followed by ACAP glucuronate. The shift to glucuronidation at increased doses has also been demonstrated in the mouse. Four hours after a 150 mg/kg oral ACAP dose was administered to mice, the urinary glucuronate conjugate was found to be greater than the sulfate conjugate (40). Fischer et al. (41) also found a greater amount of urinary glucuronate conjugate in mice after a 500 mg/kg oral ACAP dose.

A similar dose-dependence of the ratio between sulfation and glucuronidation has been demonstrated for other substrates, including harmol. Mulder et al. (42) offered several explanations for this dose-dependence, including: 1) a depletion of inorganic sulfate at the higher doses of the substrate, 2) differences in affinity of ACAP for the enzymes for sulfation and glucuronidation and 3) a non-homogenous distribution of the transferases and ACAP in the liver.

The limited availability of inorganic sulfate for incorporation into PAPS has been cited by some as the reason for the dose-dependence of the sulfation of ACAP. ACAP causes appreciable depletion of endogenous inorganic sulfate in animals and humans (43-50). Replenishment by sodium sulfate infusion increases ACAP elimination (44-47). PAPS concentrations have also been shown to be decreased after ACAP administration in rats, and serum sulfate concentration was found to be positively correlated ($r = 0.615$) with liver PAPS concentration (49). Sulfate in the plasma has been shown to be readily available for sulfation in the liver (51). Moldeus et al. (52) selectively inhibited sulfate and glucuronate conjugation by depleting the respective cofactors in vitro. However, there is also evidence that the dose-dependence of ACAP sulfate conjugation is due only in part to inorganic sulfate depletion and therefore to PAPS depletion. Weitering et al. (53) provided evidence that although there was an initial decrease in plasma sulfate after phenol doses, it was followed by a fairly rapid compensation, possibly by an increased supply from other body sites. They were able to decrease plasma sulfate only to 77% of control values in rats in one hour, yet one hour following 600 mg/kg ACAP i.p., others have shown a decrease to 30% of control (49). Mulder and Keulemans (54) have observed that in isolated perfused rat liver, as the inorganic sulfate concentration decreased, the rate of harmol sulfation decreased. They suggested that the decrease in sulfation was due to a relatively high K_m of PAPS synthesis rather than total depletion of inorganic sulfate.

The second explanation offered for the dose dependency of ACAP sulfation is that the limited capacity to conjugate ACAP at high doses by sulfation may be a reflection of the saturation of the PST. The ACAP K_m for sulfation is much lower than the ACAP K_m for glucuronidation (37). This could account for the increase in ACAP sulfate conjugation at low ACAP concentrations and an increase in ACAP glucuronate conjugation at higher ACAP concentrations. When ACAP sulfation was suppressed by an inhibitor, such as 2,6-dichloro-4-nitrophenol, in a perfused rat liver preparation (55), glucuronate and GSH conjugations were not significantly increased. The lack of a compensatory increase in glucuronidation was attributed to the high K_m for glucuronidation in relation to the ACAP concentration used. These differences in affinity may also be a partial explanation for the dose-dependence observed for ACAP conjugation by sulfate and glucuronate.

The third rationale offered for the dose dependency of ACAP sulfation involves the distribution of the conjugating enzymes in the liver. Pang et al. (56) provided evidence that sulfation enzymes are located mainly in periportal cells and glucuronidation enzymes are more concentrated in centrilobular cells. At low dose, the ACAP would be exposed first to periportal cells and when these are saturated, as in higher doses, the ACAP would be conjugated by glucuronate in the centrilobular cells. Additional study is needed to prove whether this hypothesis is valid for ACAP conjugation.

The sulfate and glucuronate conjugation pattern of harmol in rats in vivo was not affected by a 72 hour fast (57). However, a diet having 48% less methionine and cysteine than the control diet, increased the activity of UDP-glucuronosyl transferase (58).

The majority of the glucuronate and sulfate conjugates are eliminated directly by the kidney as urinary constituents. The pathways for disposition of the ACAP-GSH conjugate and its degradation products, the cysteine conjugate and the mercapturate, have been examined by Wong et al. (40) and Fischer et al. (59). Their results indicate that little of the GSH conjugate formed in the liver appears in that form in the urine. The ACAP-GSH conjugate is cleared from the liver via the bile, reabsorbed, metabolized and then excreted as the cysteine or mercapturate conjugates in the urine. Fischer et al. (59) found that the conversion of the ACAP-cysteine conjugate to the mercapturate occurred in the intestine and the kidney. Grafstrom et al. (60) showed that the enzyme, γ -glutamyltranspeptidase, is present in rat intestine and that the conversion of the GSH conjugate from bile to the cysteine form occurs during reabsorption from the intestine.

Other aspects of ACAP metabolism are species, sex and age differences and the circadian rhythm found in susceptibility to ACAP toxicity. Davis et al. (19) demonstrated that the incidence and severity of ACAP induced liver necrosis was greater in Golden Syrian hamsters and mice than in rats. Rats, guinea pigs and rabbits were relatively resistant to hepatic injury by ACAP. The excretion pattern

of ACAP-glucuronate and ACAP-sulfate after single oral doses differed in dogs and cats (61). In dogs, the ACAP-glucuronate was the principal urinary metabolite, while in cats the ACAP-sulfate conjugate was the major urinary metabolite. The ACAP-sulfate urinary concentration decreased in cats as the ACAP dose increased.

The susceptibility to ACAP-induced hepatotoxicity has been assessed by serum glutamate-pyruvate transaminase and lactate dehydrogenase activity elevations and histological evaluations in neonatal (11 days) and young rats (19 and 33 days) (62,63). A decreased susceptibility was observed in neonatal rats compared with older rats. GSH depletion was maximal at all three ages after doses ranging from 750-1250 mg/Kg and covalent binding of [14 C]ACAP to hepatic and renal macromolecules was greater in the rats 11 and 19 days old. Green and Fischer (64) found that sex-related differences in rats were more easily demonstrated at lower ACAP doses. Sex-related differences in ACAP conjugate patterns were also reported, but not until the animals reached 60 days of age.

Experiments with mice have demonstrated a circadian rhythm in hepatic GSH levels and a simultaneous effect on ACAP lethality (65,66). GSH levels were inversely related to ACAP lethality, with the peak lethality and lowest GSH concentrations observed at 6 PM, and the lowest lethality and peak GSH concentrations at 6 AM to 10 AM.

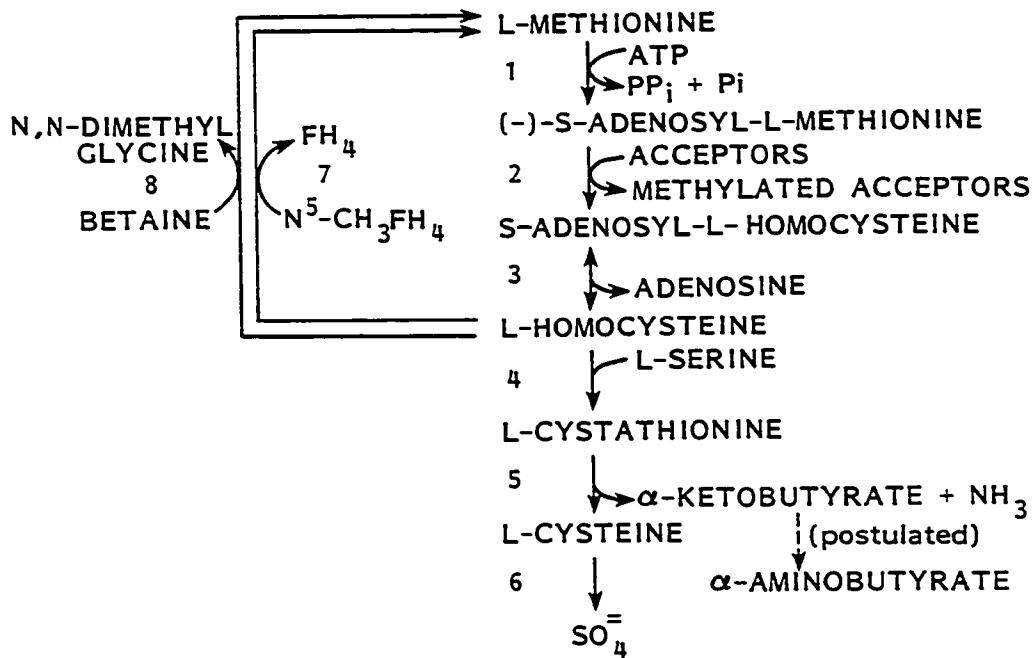
Methionine and Acetaminophen Disposition

The potential for hepatocellular necrosis increases as the GSH content of the liver is depleted by the ACAP toxic metabolite (67,68). Therefore, the GSH content is critical in protection from ACAP induced hepatotoxicity. Accordingly, the availability of the sulfur-containing amino acids, which are GSH precursors, is also crucial in detoxication processes.

The biochemistry of sulfur-containing amino acids has been reviewed recently by Cooper (69). The two pathways of methionine breakdown proposed by this review are the transsulfuration pathway (figure 2), and the transaminative pathway (figure 3). The diagrams of these pathways are presented from Benevenga and Egan (70), with some modification. The importance of the transaminative pathway versus the transsulfuration pathway in methionine catabolism is not known. However, it is generally believed that the transsulfuration pathway predominates.

The reactions involved in the transsulfuration pathway (figure 2) are now well defined. These include the transfer of sulfur from methionine to cystathionine and then cleavage by the enzyme, β -cystathionase (cystathionine synthase) to yield cysteine. The two salvage pathways for methionine illustrated in figure 2 involve the N^5 -methyltetrahydrofolate-homocysteine methyltransferase (mTHF-MT) and the betaine-homocysteine methyltransferase (BH-MT).

The control of the transsulfuration pathway was investigated by Krebs et al. (71). They concluded that the levels are controlled to a



1. S-ADENOSYLMETHIONINE SYNTHETASE
2. METHYL TRANSFERASES (MULTIPLE ENZYMES)
3. S-ADENOSYLHOMOCYSTEINE HYDROLASE
4. CYSTATHIONINE SYNTHASE
5. γ -CYSTATHIONASE
6. CYSTEINE OXIDASES (CYTOSOL + MITOCHONDRIAL)
7. N^5 -METHYLTETRAHYDROFOLATE-HOMOCYSTEINE TRANSMETHYLASE
8. HOMOCYSTEINE-BETAINE TRANSMETHYLASE

FIGURE 2. Transsulfuration pathway

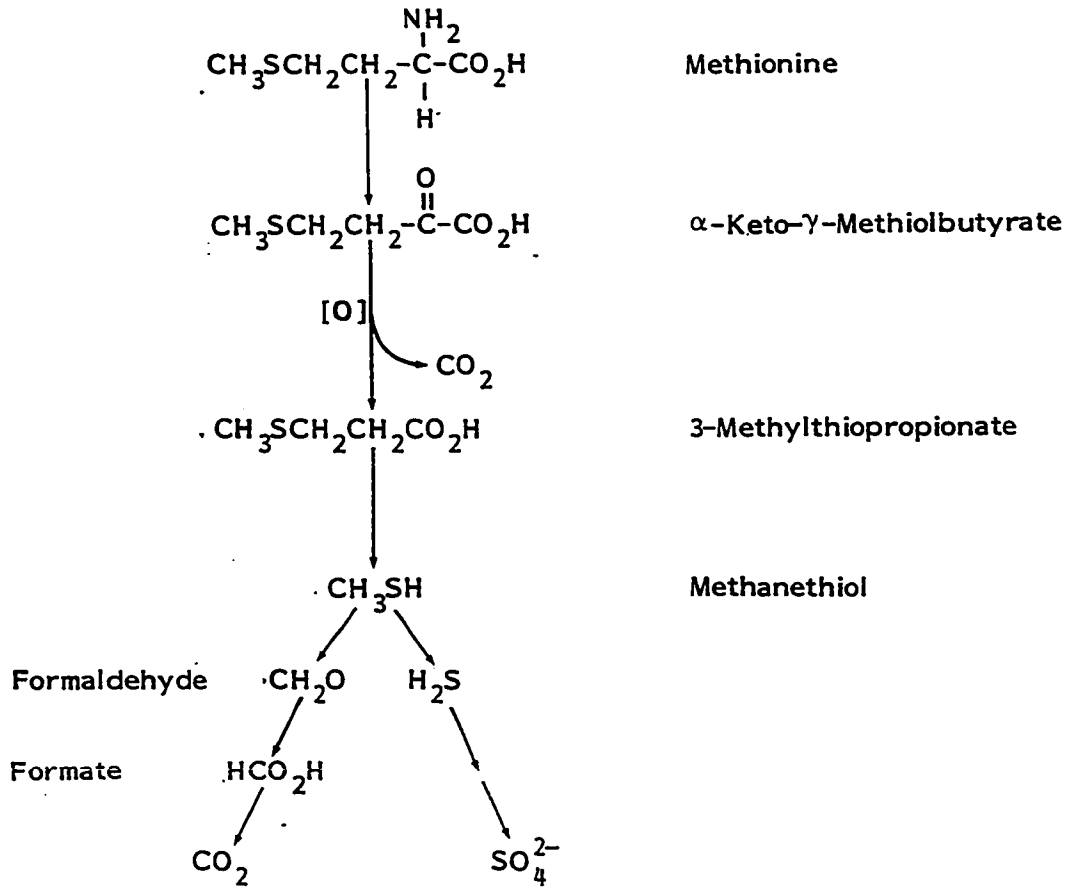


FIGURE 3. Transaminative pathway

fine degree at the homocysteine level; when there is a deficiency of methionine, homocysteine is remethylated by mTHF-MT or BH-MT, and when methionine is present in excess, homocysteine is catabolized by the cystathionine synthase reaction to yield more cysteine. These conclusions have been confirmed by Finkelstein and Martin (72). They measured the utilization of homocysteine by mTHF-MT, BH-MT, and cystathionine synthase in a rat liver homogenate system. In livers from rats that had been fed a control laboratory ration, homocysteine was distributed so that 27% was accounted for by mTHF-MT, 27% by BH-MT, and 46% by cystathionine synthase. For livers from rats that had been fed 55% casein diets, an increase in transsulfuration activity was observed. The cystathionine synthase reaction accounted for 89% of the homocysteine consumed. The low protein or 3.5% casein diets caused an increase in BH-MT activity, an 83% reduction in cystathionine synthase activity, and a decrease of 55% in the utilization of homocysteine. It was suggested that the regulation of the pathway occurs through the distribution of homocysteine between competing pathways, with S-adenosylmethionine activating cystathionine synthase.

The evidence reported for the effect of methionine supply on the formation of cysteine, therefore, provides an appreciation for the effect of methionine supply on GSH content. The level of cysteine available for incorporation into GSH is the rate-limiting factor in GSH biosynthesis. The hepatic cysteine concentration is dependent on the activity of the transsulfuration pathway, dietary intake, and endogenous

protein turnover. The nutritional relationship of methionine and cysteine involves a methionine sparing effect by cysteine, part of the dietary requirement for methionine can be replaced by cysteine.

GSH biochemistry has been extensively reviewed recently (73-77). The three amino acids in the tripeptide, GSH, are glutamate, cysteine, and glycine. The thiol-reduced form (GSH) greatly predominates over the disulfide-oxidized form (GSSG); less than 5% of the total is present as GSSG. GSH is present in animal tissues in millimolar concentrations with the highest levels found in the liver. The GSH content represents at least 90% of total nonprotein low molecular weight thiols. In detoxification reactions, GSH is a substrate for GSH peroxidase and GSH S-transferase and acts as a radical scavenger.

The GSH S-transferase enzyme catalyzes the binding of GSH to the electrophilic ACAP toxic metabolite or other activated xenobiotics. GSH peroxidase catalyzes the reaction which reduces hydrogen peroxide (H_2O_2), thereby protecting against oxygen toxicity from the H_2O_2 generated by hepatic mitochondrial oxygen consumption. GSH functions as an intracellular reductant, serves as a storage and transport form of cysteine, and is involved in the γ -glutamyl cycle for transport of certain amino acids.

The maintenance of hepatic GSH levels is crucial because GSH has a broad range of vital functions. There is a balance between the rate of synthesis, the rate of utilization and the rate of GSH export from hepatocytes.

The synthesis of GSH is a two step process involving the constituent amino acid substrates and the enzymes, γ -glutamylcysteine synthetase and GSH synthetase as shown in figure 4. GSH synthesis is regulated by feedback inhibition of γ -glutamylcysteine synthetase by GSH. GSSG reduction by GSSG reductase represents another source of GSH. Homeostasis of the hepatic GSH pool under normal conditions is maintained by a continuous turnover of GSH, involving synthesis and degradation. Degradation is accomplished by the efflux of GSH across sinusoidal and canalicular membranes, consumption by conjugation reactions, oxidation to GSSG, and enzymatic breakdown. Depletion by conjugation reactions can cause rapid synthesis of GSH and increased demand for cysteine which can be met by the highly responsive transsulfuration pathway.

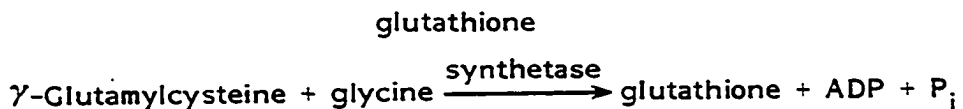
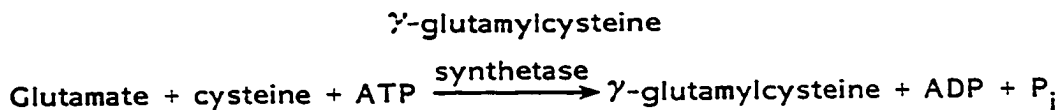


FIGURE 4. GSH biosynthesis

Hepatic cysteine content seems to be fairly constant after excess dietary methionine or cystine, the maximum change in cysteine content was from 0.1 to 0.25 $\mu\text{mole/gram}$ wet liver tissue in rats (78).

It has been suggested that there are two pools of GSH in the liver (79,80), which differ in turnover time. Meredith and Reed (81) studied the half-life of the hepatic mitochondrial and cytoplasmic pools and reported that the mitochondrial pool may represent the stable pool with the much longer half-life, compared with the cytoplasmic pool.

Hepatic GSH content is also affected by fasting (82) and diurnal variations (65,66). The extent of GSH resynthesis after ACAP challenge is dependent on many complex, interactive factors, including dietary sulfur amino acid supply.

The significance of the hepatic methionine level on ACAP disposition is apparent from the delineation of the transsulfuration pathway which incorporates the methionine sulfur into cysteine and ultimately into GSH. There have been studies completed involving manipulations of dietary methionine and cysteine and its effect on hepatic GSH content. Seligson and Rotruck (12) fed diets to rats that contained methionine in the range of 0 to 0.8% of the diet. Liver nonprotein sulfhydryl content increased with increasing dietary methionine. An increase in hepatic GSH content with increasing dietary sulfur containing amino acid content was also observed in rats by Glazenburg et al. (13). Hepatic GSH content was also shown to be increased proportionally by the amount of L-cysteine added to the diet

(83). Tateishi et al. (84) examined the proportion of dietary methionine that was incorporated into rat liver proteins or contributed its sulfur to yield cysteine and then GSH. The methionine was preferentially incorporated into liver proteins, increasing proportionally to dietary methionine increase. The methionine sulfur incorporated into GSH also increased proportionally as dietary methionine was increased from 0.18 to 0.54% of the diet. Finkelstein et al. (85) found the methionine concentration in rat liver to be relatively insensitive to dietary protein changes, while hepatic cysteine levels increased as dietary protein levels were raised. They concluded that the methionine concentration is maintained by various homeostatic mechanisms.

The methionine requirement for rats and mice has been determined previously. However, recently Neale and Waterlow (86) examined methionine oxidation and adaptation at various methionine intake levels in the rat as a basis for determining the methionine requirement. They calculated an endogenous methionine oxidation loss of 35 mg/kg body weight per day, without adaptation below the weight maintenance level.

Oxidation of sulfur amino acids is the primary source from which endogenous sulfate is derived. Inorganic sulfate in the diet accounts for a relatively small but variable portion of the endogenous sulfate. The effect of various dietary inorganic sulfate levels on rat liver inorganic sulfate concentrations was determined by Smith and Acuff (87). As dietary inorganic sulfate was raised from 0.0002 to 0.1%, liver

sulfate rose from 9.5 to 21.4 mg/g wet tissue. However, when dietary sulfate was increased to 0.42%, liver sulfate decreased. These findings led the authors to suggest that extraction of inorganic sulfate from the portal system may be enhanced by increasing dietary sulfate to the saturation level.

Thus, dietary methionine intake is important in ACAP disposition, not only as a GSH precursor, but also to serve as a sulfur source for sulfate conjugation of the ACAP reactive metabolite. An optimum nutritional status is required for an animal to maximize its defense against ACAP induced hepatic necrosis. Optimum nutritional status implies an adequate dietary supply of the essential amino acid, methionine.

Ethanol Ingestion and Acetaminophen Bioactivation

ACAP is converted to its toxic metabolite by the cytochrome P-450 dependent mixed-function oxidase (MFO) system. The activity of the MFO system, therefore, may determine in part the hepatotoxic potential of ACAP, especially when ingested in larger than recommended doses.

Inhibition of the cytochrome P-450 mediated formation of the toxic metabolite of ACAP produces a protective effect. Miners et al. (88) reported the prevention of ACAP toxicity in mice by cysteamine, which was not dependent on GSH synthesis. They suggested, but provided no direct evidence, that the most likely mechanism of action of cysteamine was inhibition of the formation of the ACAP toxic metabolite by the MFO

system. Cysteamine and cysteine in high concentrations were found to decrease covalent binding of the ACAP toxic metabolite in mouse liver microsomal incubations by Buckpitt et al. (89). Inhibition of the formation of the toxic metabolite by the MFO system could have been responsible for the observed decrease in covalent binding. The possibility that reaction of, and not formation of, the toxic product with cell components, was inhibited was not addressed. Another example of prevention of ACAP induced hepatotoxicity was observed following pretreatment of mice with the interferon inducer poly(rI.rC) (90). Decreased hepatic GSH levels and depressed cytochrome P-450 levels led the authors to suggest that the protection provided by poly(rI.rC) was the result of a decrease in toxic metabolite formation, and therefore, decreased potency of the dose to exert its cytotoxic effect. Acute ethanol administration decreased ACAP induced hepatotoxicity in rats and baboons (91,92). It was postulated that this decrease was due to the inhibition of the biotransformation of ACAP.

Induction of the cytochrome P-450 MFO system leads to increases in liver necrosis following overdoses of ACAP. Chronic ethanol ingestion potentiates the hepatic necrosis caused by ACAP in humans and laboratory animals (5-11). The extent of hepatic injury is reflected by increases in serum enzyme concentrations; serum glutamate-pyruvate transaminase (SGPT) and serum glutamate-oxaloacetate transaminase (SGOT), liver histopathology, and mortality. These results suggest that pretreatment with alcohol stimulates the cytochrome P-450 MFO system, which increases

the rate of ACAP metabolism. Studies in humans indicate that the clearance of ACAP from the plasma of chronic alcoholic subjects, or overall rate of ACAP metabolism, is increased compared with controls (93). However, there have been other mechanisms postulated for the increased hepatotoxicity of ACAP with chronic ethanol administration. Walker et al. (10) observed increased mortality, liver enlargement, and liver congestion following a toxic dose of ACAP in mice that had been pretreated with 10% ethanol in their drinking water for ten days. They suggested that the increased lethality was the result of the increased liver congestion and subsequent hypovolemia, and that the effect of ethanol on hepatocyte membranes caused the cells to be more susceptible to the ACAP toxic metabolite cytotoxic effects. The effect of chronic ethanol consumption on biliary excretion of ACAP in rats was studied by Vendemiale et al. (94). Biliary excretion of ACAP was depressed in the ethanol-fed rats and the decreases in hepatic and biliary GSH content following ACAP administration were greater than in pair fed control rats. They postulated that there are other contributions to increased ACAP induced liver pathology in rats fed ethanol chronically, such as reduced hepatic GSH levels and decreased biliary excretion.

An advance in the understanding of the mechanism of potentiation of ACAP caused liver necrosis by ethanol was brought about by the recognition of an accessory pathway of hepatic ethanol metabolism, the microsomal ethanol oxidizing system (MEOS). There are three different metabolic systems in the liver capable of oxidizing ethanol. They

include alcohol dehydrogenase (ADH) in the cytosol, catalase in the peroxisomes, and the MEOS in the endoplasmic reticulum. Figure 5 illustrates the MEOS catalyzed reaction employing O_2 and NADPH to generate water plus "active oxygen" which combines with ethanol to produce acetaldehyde and water. Activity levels of ADH and MEOS were studied in rats by Sturtevant and Garber (95). Rats maintained for 22 weeks with 15% ethanol (v/v) in the drinking water, had a notable increase in MEOS activity when compared to acute or control groups. ADH activity levels were lower and more constant in the chronic ethanol group than the acute or control groups. MEOS activity is much more prominent after chronic ethanol feeding.

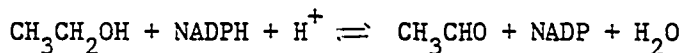


FIGURE 5. MEOS catalyzed reaction

The MEOS shares many properties with other drug metabolizing enzymes, including the involvements of cytochrome P-450, NADPH, and O_2 . The cytochrome P-450 dependency may explain the potentiation of ACAP hepatotoxicity by chronic ethanol administration.

Chronic ethanol consumption results in proliferation of the smooth endoplasmic reticulum membranes (96,97). This has been shown by subfractionation of the liver and chemical measurements of the components of rat hepatic microsomes. Also, the increase in ethanol metabolism by the MEOS is concomitant with the increases in other drug metabolizing enzymes.

Recently, a unique isozyme of cytochrome P-450, the ethanol-inducible isozyme 3a, was isolated and its catalytic activity toward ACAP was examined (98). Isozymes 3a, 4, and 6 were present in significant amounts in liver microsomes from rabbits that were given ethanol chronically. Isozymes 3a, 4, and 6 exhibited the greatest catalytic activity in a reconstituted enzyme system with ACAP as the substrate to form an intermediate capable of forming a conjugate with GSH. Phenobarbital-inducible isozyme 2 was inactive toward ACAP and isozyme 3b and 3c were 10- to 20-fold less effective than 3a, 4, or 6.

The spectral and catalytic properties of the ethanol-inducible isozyme 3a were reported by Koop and Morgan (99) and Morgan et al. (100). Cytochrome P-450 3a was isolated from rabbit liver microsomes after chronic ethanol treatment. Substrate specificity was determined for isozymes 2, 3a, 3b, 3c, and 4. Isozyme 3a had the highest activity for the oxidation of ethanol and butanol and for the p-hydroxylation of aniline.

Koop et al. (101) used antibodies to isozyme 3a to inhibit greater than 90% of the activity of purified isozyme 3a from ethanol treated rabbit liver microsomes, with aniline and ethanol as substrates, in a reconstituted system. This evidence demonstrated that isozyme 3a is responsible for most of the aniline hydroxylation and ethanol oxidation activity of microsomes from ethanol-treated rabbits.

Two potent inhibitors of ADH, imidazole and pyrazole, also induced the rabbit liver microsomal ethanol-inducible cytochrome P-450 isozyme

3a (102). The authors postulate that the cytochrome P-450 dependent MEOS might serve as a supplement to ADH under in vivo conditions.

Previous to these studies, there was much debate over the significance of cytochrome P-450 in the oxidation of ethanol. However, with the isolation, purification, and immunochemical evidence presented for cytochrome P-450 isozyme 3a in ethanol metabolism, the debate may be settled. Now, there is some question about the predominance of the cytochrome P-450 pathway over the hydroxyl radical-dependent pathway of ethanol oxidation. Krikun et al. (103) carried out studies evaluating the relative roles of these two pathways in catalyzing ethanol oxidation by microsomes from rats fed alcohol chronically and their pair-fed controls. They observed that each pathway accounted for about half of the total oxidizing activity of the microsomes, and that the activity of each was increased following ethanol pretreatment. They suggest that the ethanol-preferring cytochrome P-450 isozyme(s) actually account for only a small proportion of the total P-450 concentration. This was also indicated by Peterson et al. (8), having observed increased ACAP toxicity but no increase in cytochrome P-450 content in mouse liver following chronic ethanol consumption.

Chronic ethanol ingestion may influence the hepatotoxicity of ACAP by other means than by induction of the cytochrome P-450 MFO system. Factors that may modify liver GSH levels are of critical importance in determining the extent of ACAP toxicity. Hepatic GSH content was significantly reduced by acute and chronic ethanol administration in

rats as reported by Fernandez and Videla (104). Rats that were given an acute ethanol dose exhibited decreases in hepatic GSH and increases in lipoperoxidation, as measured by MDA formation under conditions of maximal GSH depletion (105). In isolated hepatocytes from chow-fed rats, ethanol decreased GSH, and acetaldehyde above 0.05 mM also decreased GSH (106), indicating that acetaldehyde may be responsible for the observed GSH depletion by ethanol. Hassing et al. (107), furthermore, reported an increase in hepatic GSH after treating rats with ethanol for two weeks. An increase in liver GSH content was also seen by Harata et al. (108) in rats fed 20% ethanol as the only source of drinking water for four weeks. The lipid peroxide content of the alcohol fed rats was 1.5 times that of the control animals indicating that an increase in lipid peroxide content did not necessarily lead to GSH depletion in livers of rats chronically treated with ethanol.

The manner in which ethanol is administered to the experimental animal may be important in research involving drug and nutrient interactions. Liquid diets are sometimes used involving controls which are pair fed with animals receiving ethanol isocalorically substituted for the carbohydrate source. This may lead to disruption of the normal eating pattern due to dietary restriction of the control groups. The composition of the diet may also be modified from the normal composition with carbohydrate providing a greater percentage of the total kcalories. These changes have led to alterations in enzyme activities associated with drug metabolism (109).

Dietary fat component affects the cytochrome P-450 concentration in the liver. Mounie et al. (110) found that cytochrome P-450 concentration in the hepatic microsomes of rats was increased when the rats were fed diets rich in polyunsaturated fatty acids. Variation in dietary protein was also shown to affect hepatic microsomal drug metabolism in the rat (111) that was fed ethanol chronically.

SECTION I. EFFECTS OF DIETARY METHIONINE AND ETHANOL ON ACETAMINOPHEN
HEPATOTOXICITY IN MICE

ABSTRACT

The possible interactive relationship between nutritional compromise of acetaminophen detoxification and ethanol enhancement of acetaminophen hepatotoxicity was studied in mice by using a 2- x -2 factorial design. Ethanol was administered to adult male mice at 0 or 15% solution in the drinking water, and dietary methionine levels were at 54 or 100% of the requirement. After 4 weeks, a significant reduction in the median lethal time (LT_{50}) following a high dose of acetaminophen was seen in the methionine-deficient groups. Methionine deficiency also caused a reduction in hepatic glutathione levels in the control group and in mice receiving sublethal doses of acetaminophen. PGOT levels were increased significantly by methionine deficiency but were markedly increased by the interaction of ethanol treatment and methionine deficiency. Glutathione S-transferase activity was not affected by any treatment combinations, and p-nitroanisole O-demethylase activity and relative liver weights were not increased because of chronic ethanol ingestion. These findings indicate that methionine deficiency caused glutathione reduction, which predisposes the mouse to increased acetaminophen hepatotoxicity. Ethanol consumption did not seem to potentiate the increased hepatotoxic effects caused by methionine deficiency, except as indicated by PGOT activity.

INTRODUCTION

The effect of hepatic damage attributed to the interaction of ethanol and acetaminophen is dependent upon nutritional status. Nutrition can be a critical influence on the liver's capacity for detoxification and protection from injury. In therapeutic doses, acetaminophen is rapidly metabolized in the liver by direct conjugation with UDPGA and PAPS and eliminated in the urine. However, in larger doses, the sulfation and glucuronidation pathways are overwhelmed, and acetaminophen is thought to be activated by a mixed-function oxidase involving cytochrome P-450 to a reactive toxic intermediate, which usually is complexed with reduced glutathione (GSH) within the liver to form the cysteine and mercapturate conjugates. Hepatotoxicity is enhanced if conjugation does not occur. Reactive metabolites not conjugated can covalently bind to liver macromolecules, leading to hepatic necrosis.

Repeated ethanol administration results in enhanced acetaminophen metabolism to toxic intermediates by stimulation of hepatic microsomal enzyme activities and an increase in the content of cytochrome P-450 (1,2). The toxicity of acetaminophen has been enhanced in experimental animals with ethanol as an apparent inducer of liver microsomal drug-metabolizing enzymes (3-6).

The maintenance of hepatic GSH concentration therefore is crucial for protection from acetaminophen toxicity. Glutathione concentration is the result of a dynamic system including conjugation, catabolic and

biosynthetic processes. Cysteine sources for incorporation into GSH include dietary intake, endogenous protein turnover, and the cystathionine pathway by which methionine can yield cysteine (7). The purpose of this study is to evaluate the possible interaction of dietary methionine deficiency and chronic ethanol consumption on acetaminophen toxicity and glutathione functions.

MATERIALS AND METHODS

Treatment Design

Adult male Swiss-Webster mice (Laboratory Supply Company, Inc., Indianapolis, IN) weighing 21-25 gm were randomly assigned to four treatment groups with 40 mice in each group. The animals were housed in plastic cages at 22°C with 12-hr light and dark cycle (6 AM-6 PM light) automatically maintained. The mouse was chosen as the experimental animal because it is more susceptible to acetaminophen hepatotoxicity than the rat (8).

A 2- x -2 factorial design was used, with ethanol administered at levels of 0 or 15% in the drinking water and dietary methionine levels that provided either 54 or 100% of the mouse requirement for methionine (9). The methionine-deficient (M-D) diet was composed of the basal diet (Table 1), which was prepared with soy protein (Ralston Purina Co., St. Louis, MO). The isolated soy protein contributed 2.7 gm sulfur amino acid per 100 gm protein; or 54% of the methionine requirement, a portion of which is replaceable by L-cystine. The basal diet was supplemented with L-methionine (Grand Island Biological Co., Grand Island, NY) to prepare the methionine-sufficient (M-S) diet, which provided 100% of the methionine requirement; i.e., methionine at the 0.5% level in the diet. The animals were allowed free access to food and drinking water. Food and water consumption and body weight were recorded every third day.

Table 1. Composition of basal diet

Ingredients	%	Amino acid Composition of soy protein	gm/100 mg protein	Vitamin mix	mg/kg diet
Isolated soy protein	10.12	Alanine	4.2	D-biotin	0.200
Cornstarch	36.44	Arginine	7.5	Choline chloride	750.000
Sucrose	36.44	Aspartic acid	11.9	Folic acid	0.450
Cellulose	5.00	Cysteine	1.3	Niacin	22.500
Corn oil	5.00	Glutamic acid	20.9	D-Calcium pantothenate	37.500
Safflower oil	1.00	Glycine	4.2	Pyridoxine-HCl	22.500
Vitamin mix	1.00	Histidine	2.8	Riboflavin	7.500
Mineral premix ^a	5.00	Isoleucine	4.9	Thiamine-HCl	22.500
Total	100.00	Leucine	8.2	Vitamin B ₁₂	0.023
		Lysine	6.4	(0.1% Trituration with mannitol)	
		Methionine	1.4	Vitamin D ₃	1.100
		Phenylalaine	5.3	(Activated 7-dehydrocholesterol)	
		Proline	5.7	Vitamin E	32.000
		Serine	5.1	(DL- α -tocopherol acetate)	
		Threonine	3.8	Vitamin A palmitate	2.200
		Tryptophan	1.4	Vitamin K	18.000
		Tyrosine	3.9	(Menadione)	
		Valine	4.8		

^aWilliams-Briggs mineral premix + gm/kg of diet: $ZnCO_3$ 0.03; Na_2SeO_3 0.0027; $CrCl_3 \cdot 6H_2O$ 0.0103.

All animals were subjected to treatment conditions for 4 weeks. Twelve hours before sacrifice or acetaminophen injection, the 15% ethanol solution was replaced with distilled water. Sacrifice and acetaminophen administration were timed to minimize any effect on GSH content due to the diurnal variation in hepatic GSH concentration. According to Jaeger et al. (10), the maximum hepatic GSH concentration in rats occurs between 7 AM and 1 PM. Acetaminophen injections were administered between 7 and 8 AM and control animals were sacrificed between 7 and 8 AM.

Median Lethal Time (LT_{50})

The dose dependence and time relation of acetaminophen-induced hepatotoxicity had been assessed in a preliminary experiment by giving varied doses to mice that had been pretreated in the same manner. A lethal dose for all treatment groups within an 8- to 12-hr period was estimated to be 800 mg/kg body weight (BW) by recording time and mortality rate after that dose was given. Acetaminophen (ACAP) (Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally (i.p.) as a basic solution (pH 11.0) in physiological saline at a dose of 800 mg/kg BW in ten mice from each group. The mortality time was recorded, and the LT_{50} was calculated from linear regression lines.

Determination of Hepatic Glutathione Content and Enzyme Activities

The GSH content was determined in liver from the remaining mice, which were divided into three groups; 1) mice that had received 100 mg/kg BW ACAP i.p. 12 hr before sacrifice, 2) 300 mg/kg BW ACAP i.p. 1 hr before sacrifice, and 3) from controls that had been pretreated but did not receive ACAP. All animals were killed by decapitation; the livers were removed, placed in liquid N₂, and stored at -80°C until GSH determination was done. The GSH analysis was done using Ellman's reagent (11) and the spectrophotometric method outlined by Jollow et al. (12). There were less than ten animals per group at the time of sacrifice because of mortality during the 4-week treatment period.

Blood was collected in heparinized tubes from the mice that had received a 100 mg/kg BW i.p. dose of ACAP 12 hr earlier. A reagent kit (Sigma Chemical Co., St. Louis, MO) based on the method of Karmen (13) was used to determine plasma glutamic-oxaloacetic transaminase (PGOT) activity.

A portion of fresh liver from control mice was retained for the measurement of GSH-S-transferase activity and p-nitroanisole O-demethylase activity. A microsomal preparation was used for the determination of p-nitroanisole O-demethylase activity according to Mazel (14). The GSH-S-transferase-specific activity was measured in the microsomal supernatant by using the method of Booth et al. (15) and by adapting the $\Delta\epsilon$ for 1,4-dichloro-2,4-dinitrobenzene from Habig et al. (16). Protein determinations were made according to the Lowry method (17).

Statistical Procedures

Statistical significance for the LT_{50} data was assessed by median differences, using 95% confidence limits based on z-values (18). All other data were subjected to analysis of variance. Data are shown as the mean \pm SEM.

RESULTS

Table 2 and Figure 1 illustrate the effects of methionine deficiency and ethanol consumption on food intake and growth rate. There was an initial weight loss during the first 3-4 days in all treatment groups except the M-S, 0% EtOH group. Food intake was constant in all groups except the M-D, 15% EtOH group, which exhibited a reduced intake due to an interactive effect of methionine deficiency and ethanol consumption ($P < 0.05$). Growth rate was retarded and final body weight was reduced due to methionine deficiency ($P < 0.0001$).

Table 2. Effects of treatment conditions on food consumption and final body weight

Treatment condition	N	Food consumption ^a (gm/day)	Final body weight ^b (gm)
M-D, 0% EtOH	8	4.9 ± 0.2	29.5 ± 0.8
M-S, 0% EtOH	8	4.9 ± 0.1	33.9 ± 0.5
M-D, 15% EtOH	7	4.3 ± 0.2	28.3 ± 0.7
M-S, 15% EtOH	7	4.9 ± 0.2	34.0 ± 0.9

^a_p < 0.05 for methionine and EtOH interaction.

^b_p < 0.0001 for methionine.

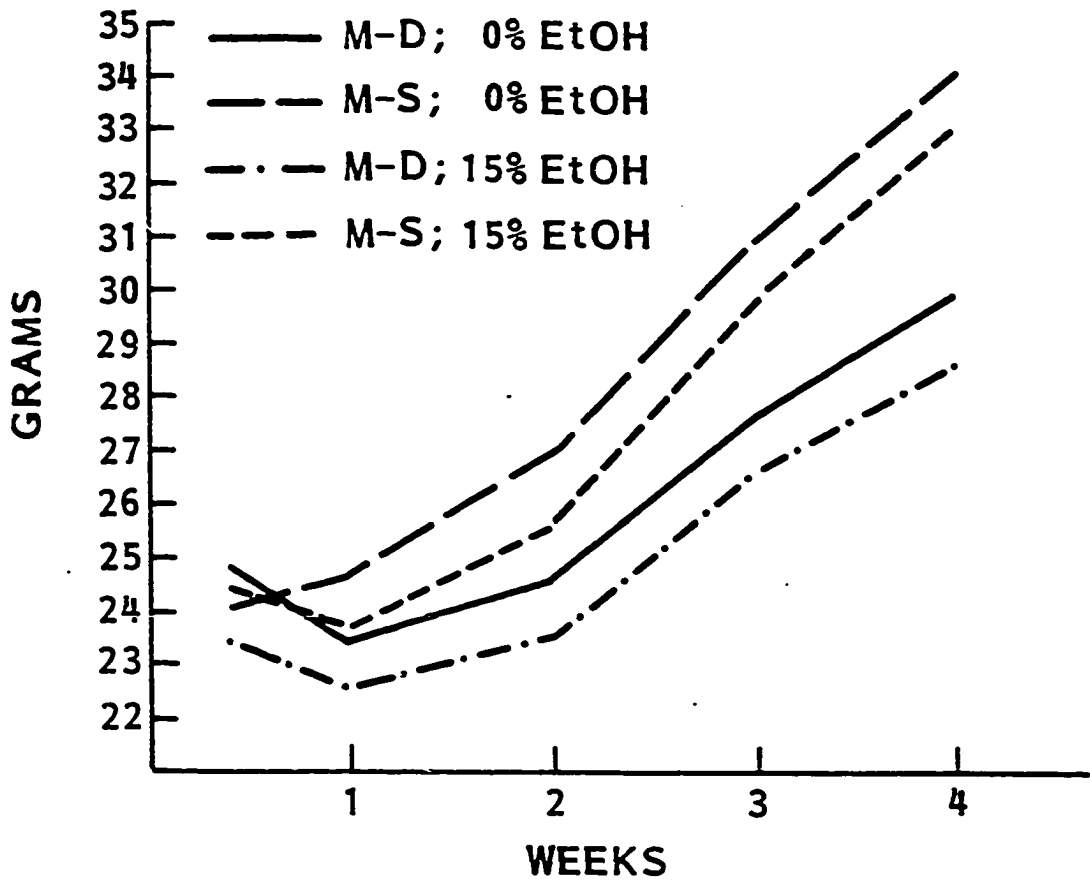


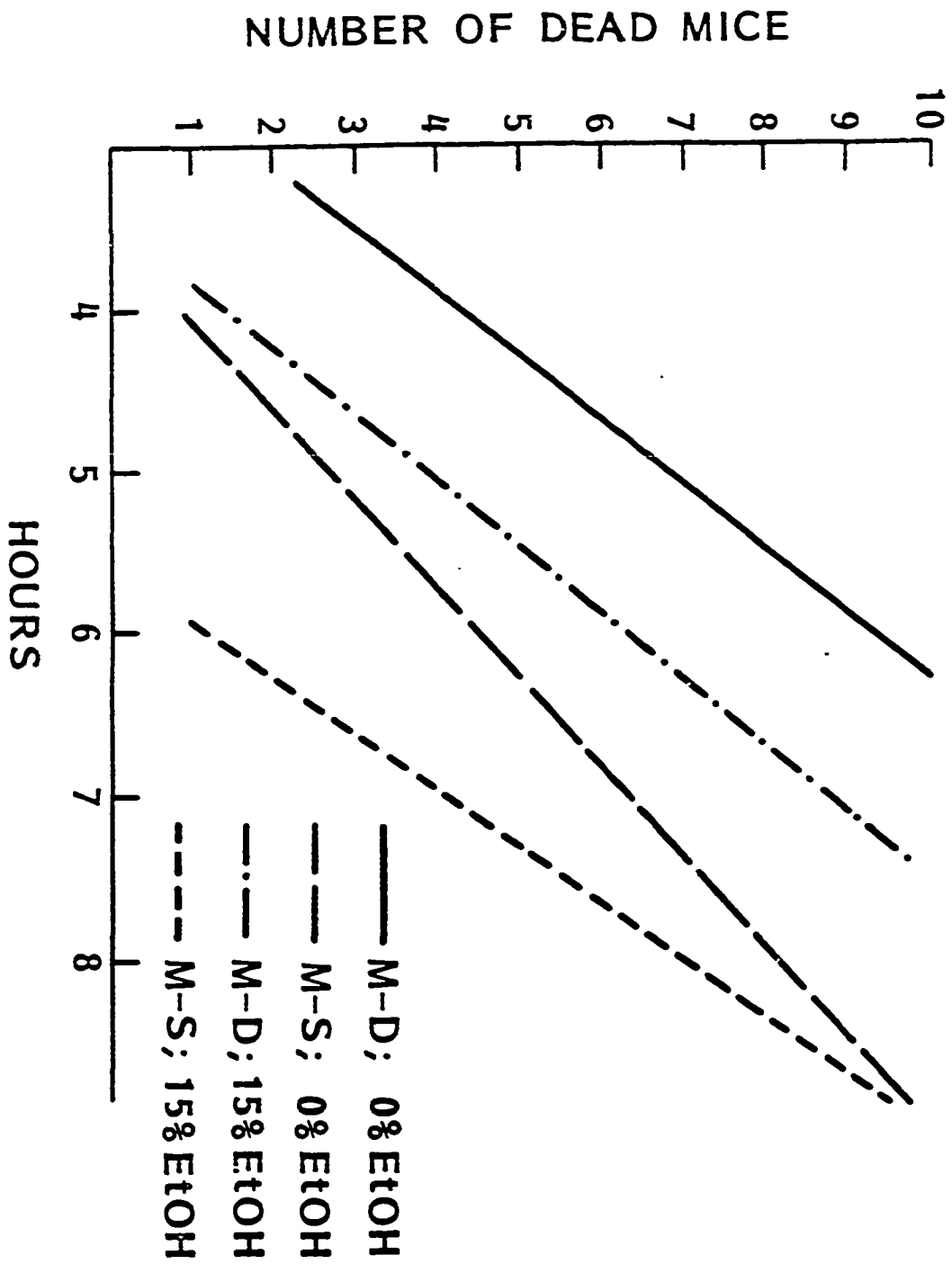
FIGURE 1. Mean growth rate in grams per days during the 4-week pretreatment period

The LT_{50} determination for all groups (Figure 2) showed that the LT_{50} was reduced with methionine deficiency ($P < 0.05$). The M-S, 0% EtOH group seemed to have a shorter LT_{50} than the M-S, 15% EtOH group, but this apparent protective effect of ethanol was not statistically significant. The effect may be real, but it is not proved by these data.

The GSH content (Figure 3) was reduced under the three circumstances in which it was determined by methionine deficiency ($P < 0.0001$ for control and 12 hr post 100 mg/kg BW i.p. ACAP and $P < 0.05$ for 1 hr post 300 mg/kg BW i.p. ACAP). In the control group, chronic ethanol ingestion increased GSH content in the methionine sufficient dietary treatment ($P < 0.01$). A 300 mg/kg BW dose of ACAP drastically depleted GSH content; 1 hr post injection, levels were 20-30% that of controls. Twelve hours after a 100 mg/kg BW dose, GSH content was 120% of the levels in controls for M-D groups and only 60-75% that of controls for the M-S groups. The M-D control and injected groups always had GSH levels lower than the comparable M-S groups.

An increase in PGOT activity (Table 3) was seen in the methionine-deficient mice ($P < 0.01$); however, a more dramatic increase was seen in the M-D, 15% EtOH group because of the interaction of methionine deficiency and chronic ethanol ingestion ($P < 0.05$). Ethanol also increased PGOT activity ($P < 0.05$) in methionine-deficient or methionine-sufficient mice.

FIGURE 2. LT_{50} after 800 mg/kg BW acetaminophen administration via intraperitoneal injection



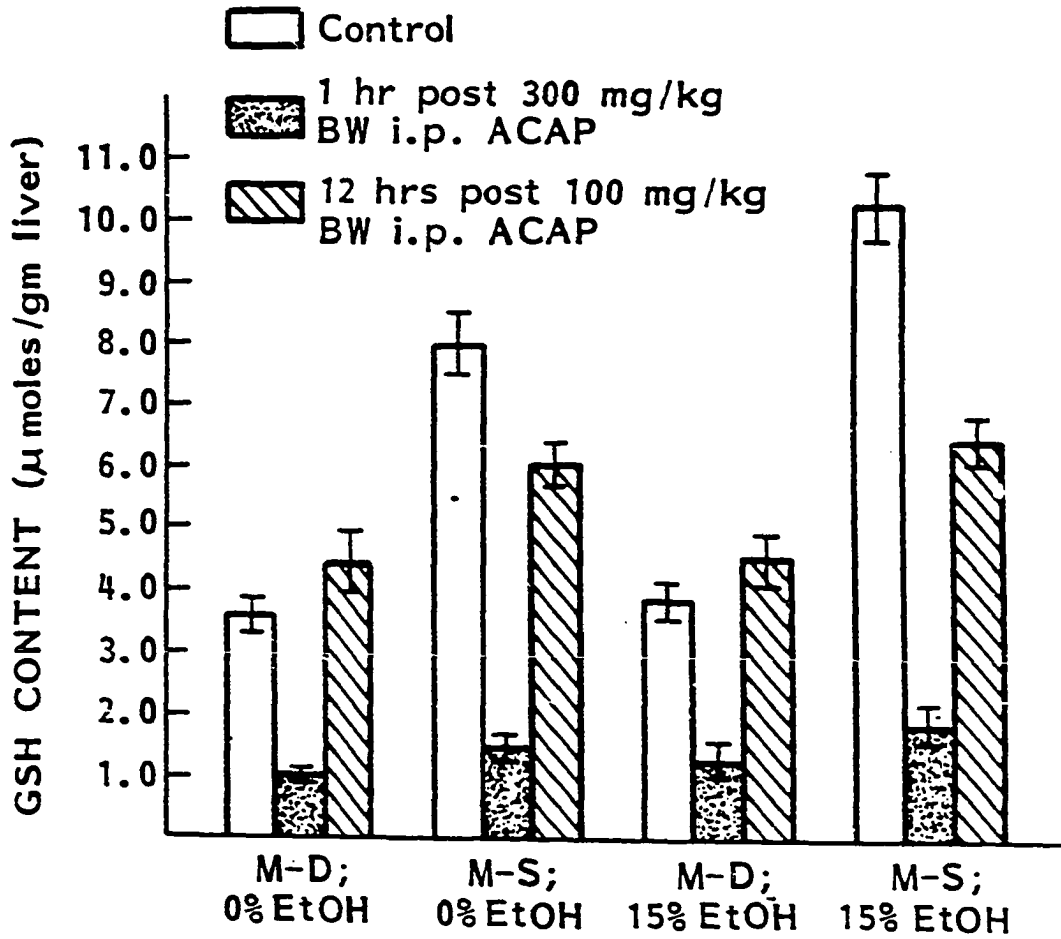


FIGURE 3. Hepatic glutathione concentrations after intraperitoneal administration of acetaminophen

Table 3. PGOT activity 12 hrs after 100 mg/kg BW i.p. acetaminophen

Treatment condition	N	(μ moles/min/l) ^{a,b,c}
M-D, 0% EtOH	6	424 \pm 95
M-S, 0% EtOH	5	160 \pm 20
M-D, 15% EtOH	8	1858 \pm 418
M-S, 15% EtOH	7	200 \pm 13

^a_p < 0.01 for methionine.

^b_p < 0.05 for EtOH.

^c_p < 0.05 for methionine and EtOH interaction.

In controls, GSH-S-transferase activity (Table 4) was not significantly altered due to differences in treatment conditions when expressed as activity per mg protein or per g of liver. Table 5 presents the p-nitroanisole O-demethylase activity and the liver weight expressed as percentage of total body weight. Both measurements were found to be greater (P < 0.05) due to methionine adequacy but not changed by chronic ethanol consumption.

Table 4. Effects of treatment conditions on GSH-S-transferase activity without i.p. acetaminophen

Treatment condition	N	GSH-S-transferase activity ^a (μ moles/min/mg protein)
M-D, 0% EtOH	8	152.6 \pm 16.4
M-S, 0% EtOH	8	141.3 \pm 13.9
M-D, 15% EtOH	7	167.7 \pm 24.3
M-S, 15% EtOH	7	115.3 \pm 12.2

^aNot significantly different.

Table 5. Effects of treatment conditions on p-nitroanisole O-demethylase activity and relative liver weights of mice without i.p. acetaminophen

Treatment condition	N	p-nitroanisole O-demethylase activity ^a (μ moles/hr/mg protein)	Liver weight \div body weight \times 100 (%) ^a
M-D, 0% EtOH	8	0.022 \pm 0.002	4.8 \pm 0.2
M-S, 0% EtOH	8	0.023 \pm 0.002	5.3 \pm 0.1
M-D, 15% EtOH	7	0.019 \pm 0.002	4.9 \pm 0.3
M-S, 15% EtOH	7	0.027 \pm 0.003	5.8 \pm 0.3

^ap < 0.05 for methionine.

DISCUSSION

Growth was not severely compromised by methionine deficiency. Both the methionine-deficient and methionine-sufficient mice fell within the range of average weights for 26 inbred mouse strains compiled by Poiley (19), but the deficient mice did have reduced weights compared with the methionine-sufficient (Table 2).

Methionine deficiency compromised survival time after treatment with a lethal dose of ACAP (Figure 2). Methionine-adequate groups showed a longer survival time or greater protection against ACAP-induced mortality. Although the exact mechanism of death is unknown (20), it seems likely that, if hepatotoxicity is decreased due to greater GSH stores, survival time after a lethal dose would be increased. The decrease in the LT_{50} probably coincides with a reduction in GSH content for methionine-deficient animals. A direct relationship of hepatic GSH availability and extent of ACAP hepatotoxicity has been extensively documented (21,22).

Methionine deficiency was found to be responsible for reduced hepatic GSH concentrations both before and after ACAP challenge (Figure 3). Hepatic GSH concentration was also decreased due to lowering of the cysteine/methionine content of the diet of the rat (23).

The amount of drug-GSH conjugates excreted as mercapturic acids can exceed by several-fold the amount of GSH present in the liver before the drug is administered (24). This indicates that the liver is capable of synthesizing large amounts of GSH at a rapid rate when needed. It would

seem that a methionine-deficient animal would have some difficulty meeting the increased demand for cysteine for rapid GSH biosynthesis. However, the methionine-deficient ACAP-treated groups had GSH levels that were 120% of that of the control, and methionine-sufficient animals generated only 60-75% of that of control by 12 hr after a 100 mg/kg BW i.p. dose of ACAP.

Control animals treated with ethanol had an increase in GSH content with methionine adequacy. Other researchers have shown a decrease in hepatic GSH content following chronic ethanol ingestion (25-27). However, Hassing et al. (28) found that rats receiving 36% of their calories as ethanol for two weeks had elevations in hepatic reduced and oxidized glutathione. Additional study of the mechanism for regeneration of the GSH pool after stress in nutritionally compromised animals is needed.

The PGOT activity was markedly increased due to the interaction of methionine deficiency and ethanol treatment (Table 3). This finding was not associated with interactive effects on GSH levels; the M-D, 15% EtOH group did not have the lower GSH content in any of the three treatment circumstances under which it was determined. However, there was an increase in PGOT levels attributable to methionine deficiency.

The GSH-S-transferase activity did not respond to dietary methionine or ethanol treatment (Table 4). Induction of GSH transferase is well documented after phenobarbital administration, although other compounds are also inducers (29). In this case, ethanol was not

effective as an inducer of GSH-S-transferase. It also seems that methionine and GSH are not limiting factors in GSH-S-transferase activity.

The potentiation of ACAP hepatotoxicity by chronic ethanol consumption has been observed in the mouse as well as the rat. Enhanced hepatotoxicity after chronic ethanol consumption was shown by a decrease in the LD₅₀ (3,4), and increase in GOT activity (4-6), and greater GSH depletion after ACAP treatment than controls, which received ACAP but no ethanol (4,5). The proposed mechanism for the increased hepatotoxicity seen is enhanced production of reactive metabolite due to ethanol induction of cytochrome P-450 content and drug-metabolizing enzymes. Chronic ethanol consumption results in proliferation of smooth endoplasmic reticulum membrane, which is associated with a concomitant increase in microsomal capability for activation of drugs such as isoniazid and ACAP (30).

However, p-nitroanisole O-demethylase activity and the relative liver weights of ethanol-treated mice were not greater than those of mice not given ethanol. These parameters differed only due to methionine content of the diet (Table 5) and did not provide evidence for induction of microsomal activation of ACAP by ethanol. The difference in p-nitroanisole O-demethylase activity due to methionine content was more evident in the groups fed ethanol. It seems reasonable to propose that, since protein deficiency decreases hepatic drug-metabolizing ability (31), methionine deficiency could have similar

effects, and could exert a protective effect by causing a reduction in the formation of the ACAP toxic metabolite. Evidence for this effect could not be determined from our data.

In summary, it can be concluded that methionine deficiency caused GSH reduction that predisposed the mouse to increased ACAP hepatotoxicity and that ethanol potentiated the hepatotoxic effects of ACAP.

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SECTION II. EFFECTS OF DIETARY METHIONINE AND ETHANOL ON ACETAMINOPHEN
CONJUGATION IN MICE

ABSTRACT

Adult, male Swiss-Webster mice were pair-fed an ethanol-containing liquid diet for 4 weeks that provided 46 or 100% of the methionine requirement in a 2- x -2 factorial design. Hepatic microsomal protein, relative liver weight, and microsomal aniline hydroxylase activity were increased in the ethanol-fed groups. Following a 300 mg/kg BW i.p. acetaminophen (ACAP) dose, serum inorganic sulfate and hepatic reduced glutathione (GSH) concentrations were decreased, while uridine diphospho-glucuronosyl transferase activity was not changed. GSH levels were reduced to a greater extent in the methionine-deficient groups. Data generated from experiments incorporating [^{14}C]-methyl-methionine, [^{35}S]-methionine, and $\text{Na}_2[^{35}\text{S}]\text{O}_4$ into hepatic proteins did not reflect differences in transsulfuration activity as a result of dietary methionine deficiency. Endogenous hepatic methionine was decreased after ACAP administration. Methionine deficiency decreased the distribution of ACAP into the urinary sulfate conjugates, and ethanol consumption increased the percentage of sulfate and mercapturic acid conjugates formed. These results indicate that methionine deficiency compromises the normal pathways of ACAP disposition in the mouse and that chronic ethanol ingestion potentiates this compromise by increasing the amount of activated ACAP formed.

INTRODUCTION

Acetaminophen (ACAP) ingestion imposes nutritional demands which primarily involve the maintenance of hepatic glutathione (GSH) levels. GSH is necessary for conjugation of the ACAP toxic metabolite generated by hepatic cytochrome P-450 dependent oxidation after larger than recommended doses. Induction of hepatic microsomal oxidation by ethanol increases the production of activated ACAP and thus, the demand for cysteine, the rate-limiting precursor for GSH biosynthesis. Methionine sulfur is donated to yield cysteine via the hepatic transsulfuration pathway. Sulfur-containing amino acids also serve as a source for sulfate for ACAP-sulfate conjugate formation.

Dietary methionine deficiency has been associated with decreased hepatic GSH levels in rats (1,2). Chronic ethanol administration has been shown to cause increased ACAP induced hepatotoxicity in man and laboratory animals (3-9). The purpose of this study is to determine how dietary methionine inadequacy and chronic ethanol consumption interact to affect parameters involved in the detoxification of activated ACAP and the pattern of urinary ACAP conjugates formed.

MATERIALS AND METHODS

Treatment Design

Adult, male Swiss-Webster mice (Laboratory Supply Company, Inc., Indianapolis, IN) weighing 21-25 gm were randomly assigned to four groups with 12 mice in each group for each of three experiments. The animals were housed in hanging steel mesh cages at 22°C with a 12-hr light and dark cycle (6 AM - 6 PM light) automatically maintained.

A 2- x -2 factorial design was used with two levels of dietary methionine (met) and two levels of ethanol (EtOH). The animals were pair-fed a liquid diet (Table 1) (10) for four weeks which provided the following levels of met and EtOH: met deficient or 46% of the mouse requirement for met (11) without EtOH (M-D); met sufficient or 100% of the requirement without EtOH (M-S); met deficient with 25% of the kcalories isocalorically substituted by EtOH (M-D; EtOH); and met sufficient with EtOH (M-S; EtOH). Food consumption was recorded daily and the animals were weighed weekly.

Twelve hours before ACAP administration or sacrifice, the liquid diet was replaced with distilled water. ACAP injections and the sacrifice of the mice were always carried out between 8 AM and 9 AM to avoid variation due to the diurnal pattern of GSH concentration (12).

Table 1. Composition of the liquid diet

Component	Amount g/L
Isolated soy protein ^a	26.25
Dextrose ^b	188.00
Cellulose powder ^b	10.00
Safflower oil ^b	2.20
Corn oil ^c	10.80
Vitamin mixture ^d	12.50
Mineral mixture ^e	10.00
Xanthan gum ^f	2.00
Distilled-deionized water	880.00

L-methionine added to M-S diets to provide 100% of requirement

^aSuppro 660, Ralston Purina Co., St. Louis, Missouri.

^bTeklad Test Diets, Madison, Wisconsin.

^cMazola Brand, Iowa State University Food Service, Ames, Iowa.

^dThe vitamin mix provided the following in grams/kg mixture: d-biotin 0.02; choline chloride 75.0; folic acid 0.045; niacin 2.25; d-calcium pantothenate 3.75; pyridoxine - HCl 2.25; riboflavin 0.75; thiamin-HCl 2.25; vitamin B₁₂ (0.1% trituration with mannitol) 0.0023; vitamin D₃ (activated 7-dehydrocholesterol) 0.11; vitamin E (dl- α -tocopherol-acetate) 3.2; vitamin A palmitate 0.22; vitamin K (menadione) 1.8. Made to 1 kg with dextrose.

^eWilliams-Briggs mineral mix with Na₂SeO₃, 0.055 g; ZnCO₃, 0.55 g; and CrCl₃·6H₂O, 0.205 g added/kg mixture.

^fKeltrol, Kelco Corp., Clark, New Jersey.

Experiment 1. GSH, Sulfate and Enzyme Activities

Half of the animals in each group were given 300 mg/kg BW i.p. ACAP (Sigma Chemical Co., St. Louis, MO) dissolved in 40% propylene glycol/physiological saline one hour before sacrifice. The remaining mice were given the vehicle only as an i.p. injection. Blood was collected after decapitation and centrifuged for serum inorganic sulfate determination according to the turbidimetric method of Berglund and Sorbo (13) as modified by Krijgheld et al. (14). Hepatic GSH content was measured using Ellman's reagent (15) according to Jollow et al. (16). Liver microsomes were prepared and used for the determination of aniline hydroxylase activity by spectrophotometrically measuring the product, p-aminophenol, according to Mazel (17). Uridine diphosphoglucuronosyl transferase (UDPGT) activity was assayed in liver microsomes according to the spectrophotometric method of Hollman and Touster (18) which measured the conjugation of p-nitrophenol. Protein content in all experiments was determined by the method of Lowry et al. (19).

Experiment 2. In Vitro Protein Synthesis

In the second experiment, half of the mice in each group were also given 300 mg/kg BW i.p. ACAP one hour before sacrifice. Livers were removed, weighed and immediately placed on ice. Samples were minced and then homogenized with 5 strokes using a Potter-Elvehjem homogenizer with a teflon pestle in 4 volumes of ice-cold buffer. The homogenizing

buffer was of the following composition: 0.25 M sucrose, 0.25 M KCl, 0.005 M Mg Acetate and 0.05 M Tris-HCl buffer at pH 7.6. The homogenate was then centrifuged at 6,900 g for 10 minutes. The post-mitochondrial supernatant (PMS) was used for L-[¹⁴C]-methyl-met, L-[³⁵S]-met, and Na₂³⁵SO₄ incorporation into hepatic proteins according to the method of Nakano and Sidransky (20) with some modifications. The incubation system contained reagents in the following concentrations: 77 mM KCl, 48 mM NaCl, 19.3 mM Tris-HCl (pH 7.5), 4.9 mM MgCl₂, 0.08 mM 2-mercaptoethanol, 1.2 mM ATP, 0.88 mM GTP, 30 μg pyruvate kinase, 9.6 mM phosphoenolpyruvate and 0.05 mM each of 18 amino acids (except sulfur containing amino acids). Reactions were carried out with PMS representing either 0.01 or 0.02 gm liver, the incubation buffer and either 0.5 μCi L-¹⁴Cmethyl-met (sp. act. 51 mCi/mmole), 0.25 μCi L-³⁵S-met (sp. act. 1080 Ci/mmole) both purchased from Research Products International Corp., Mount Prospect, IL or 0.5 μCi Na₂³⁵SO₄ (sp. act. 73.3 mCi/mmole) from Amersham Corp., Arlington Heights, IL. The amount of exogenous L-met added was kept constant in each incubation mixture. Incubation, protein preparation and detection measures were done according to Nakano and Sidransky (20) without modification. Endogenous free methionine and cysteine/cystine were assayed in the PMS after protein precipitation with equal volumes 6% sulfosalicylic acid by automated ion exchange amino acid analysis (Durrum D-400 Amino Acid Analyzer, Iowa State University Department of Biochemistry and Biophysics).

Experiment 3. Urinary ACAP Conjugates

All animals in each group were given p-[³H(G)]-hydroxyacetanilide (sp. act. 17.3 Ci/mole, New England Nuclear, Boston, MA) at 150 µCi/kg BW with cold ACAP added to provide 300 mg/kg BW i.p. ACAP and L-³⁵S-cysteine (sp. act. 1490 Ci/mole, Amersham Corp., Arlington Heights, IL) at 67 µCi/kg BW. Exogenous cysteine was negligible compared to endogenous hepatic cysteine. Urine was collected over dry ice for 8 hrs and stored at -80°C until assayed for ACAP conjugate formation. The HPLC method of Howie et al. (21) was used to estimate urinary ACAP and its sulfate, glucuronide, and mercapturic acid conjugates with the following modifications. A Beckman 110A high-performance liquid chromatograph equipped with a Hitachi 100-10 spectrophotometric UV variable wavelength detector, a Beckman recorder and a Columbia Scientific Industries Supergrator-I integrator was used with a Gilson fraction collector and a Spherisorb ODS-2 10µ column (250 mm length x 4.6 mm I.D.). Urine was diluted 5-fold, filtered (Rainin Instrument Co., Woburn, MA) and a 5 µl volume was injected. A representative chromatograph with ³⁵S and ³H activity is given in Figure 1. Aliquots of urine were treated with β-glucuronidase (Sigma Type VII from *Escherichia coli*) or sulfatase (Sigma, Type H-2 from *Helix pomatia*) to identify the glucuronide and sulfate conjugate peaks. The peak remaining with ³⁵S activity after sulfatase treatment was identified as the ACAP-mercapturic acid peak. The free ACAP peak was identified by comparison with reagent grade ACAP elution time and peak.

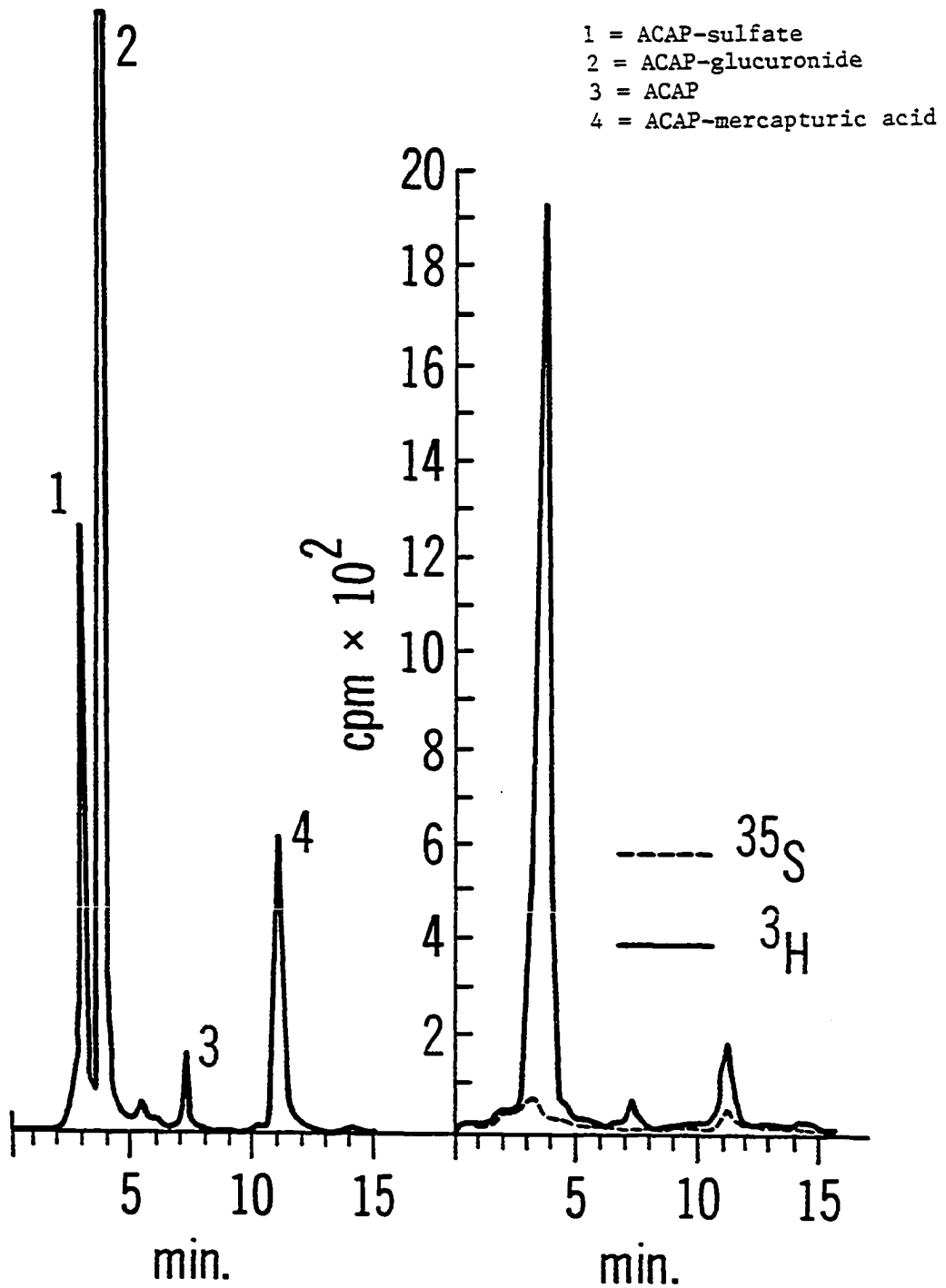


FIGURE 1. Representative chromatogram with corresponding sulfur-35 and tritium activity from 8-hr urine from mice after 300 mg/kg BW i.p. ACAP

Statistical Procedures

All data were subjected to analysis of variance and are presented as the mean \pm SEM.

RESULTS

Pair-feeding kept the intake of all 4 groups fairly constant as shown in Figure 2. There was an initial decrease as ethanol was increased to provide from 10% to 25% of energy intake during the first 2 weeks. The effect of dietary methionine deficiency and chronic ethanol consumption on weight gain is illustrated by Figure 3 and Table 2. A decrease in body weight was seen after the initial gradual increase in dietary ethanol, followed by a steady rise until the end of the pretreatment period. At this time, body weight was significantly lower in the M-D groups and was significantly less due to ethanol in the liquid diet.

Experiment 1

The results in Table 3 and Figures 4 and 5 demonstrate the effect of chronic ethanol consumption on parameters which may indicate induction of drug-metabolizing enzyme activity. Microsomal protein, liver weight expressed as percent BW or relative liver weight, and hepatic microsomal aniline hydroxylase activity were all increased significantly in the EtOH-consuming groups.

Serum inorganic sulfate (Table 4) and hepatic GSH concentrations (Figure 6) were both decreased after ACAP administration, however only the GSH content was decreased in the M-D groups. ACAP administration had no effect on UDPGT activity, but there were decreases in activity in the M-D groups when expressed as change in absorbance per hour per liver

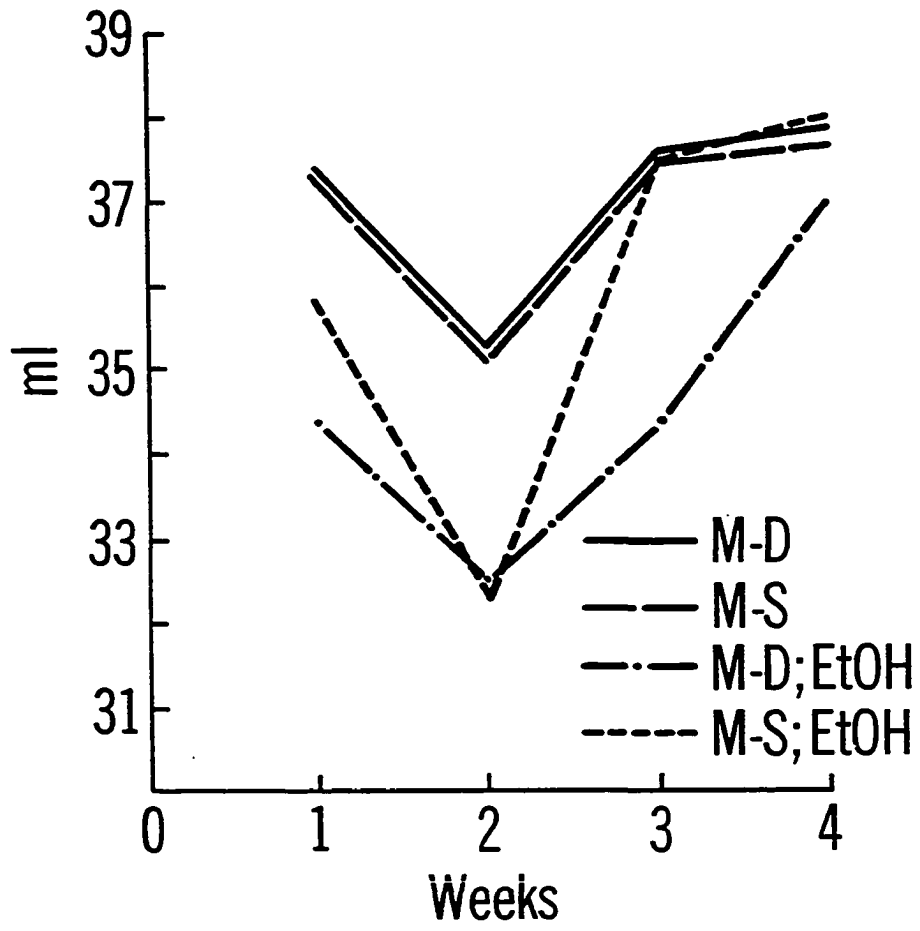


FIGURE 2. Mean liquid diet consumption (ml) per week by pair-fed mice

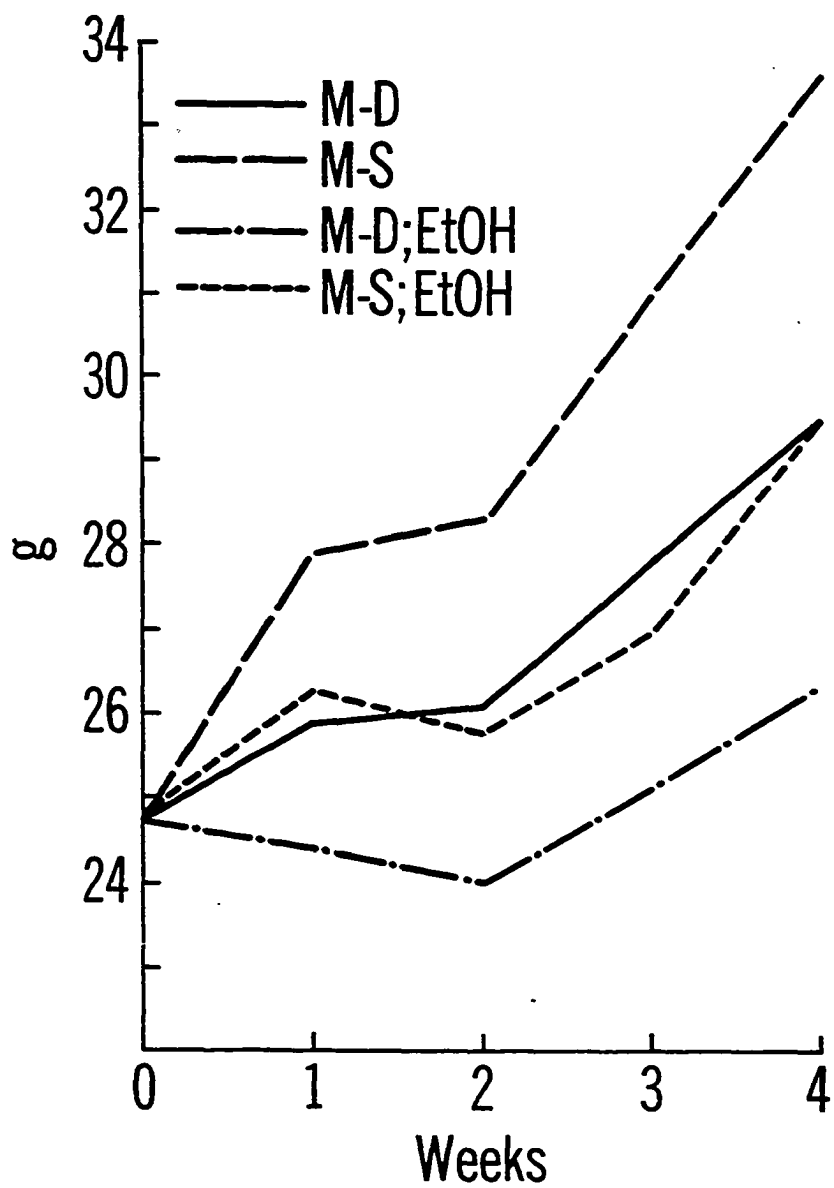


FIGURE 3. Mean weight gain during a 4-week pretreatment period

Table 2. Effect of diet on final body weight

Treatment	Body weight g ^{a,b}	N
M-D	31.56 ± 0.57	36
M-S	34.71 ± 0.53	36
M-D; EtOH	27.56 ± 0.64	36
M-S; EtOH	30.92 ± 0.59	36

^a_p < 0.0001 for met.

^b_p < 0.0001 for EtOH.

Table 3. Effect of methionine deficiency and EtOH on hepatic protein content

Treatment	Mg microsomal pro/g liver ^a	N	Mg PMS supernatant pro/g liver ^b	N
M-D	31.32 ± 1.99	12	147.81 ± 2.86	10
M-S	30.24 ± 1.33	12	141.34 ± 5.84	9
M-D; EtOH	36.94 ± 2.63	12	138.64 ± 3.44	11
M-S; EtOH	34.71 ± 1.04	12	152.05 ± 3.34	10

^a_p < 0.01 for EtOH.

^b_p < 0.01 for met*EtOH.

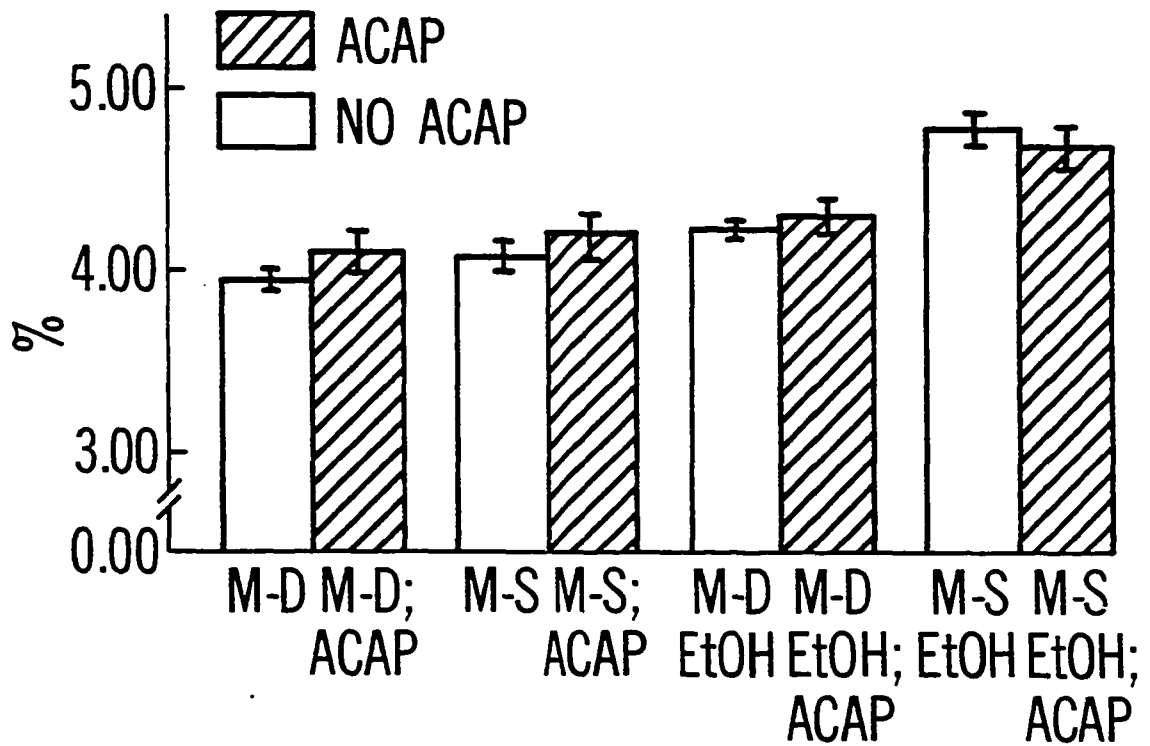
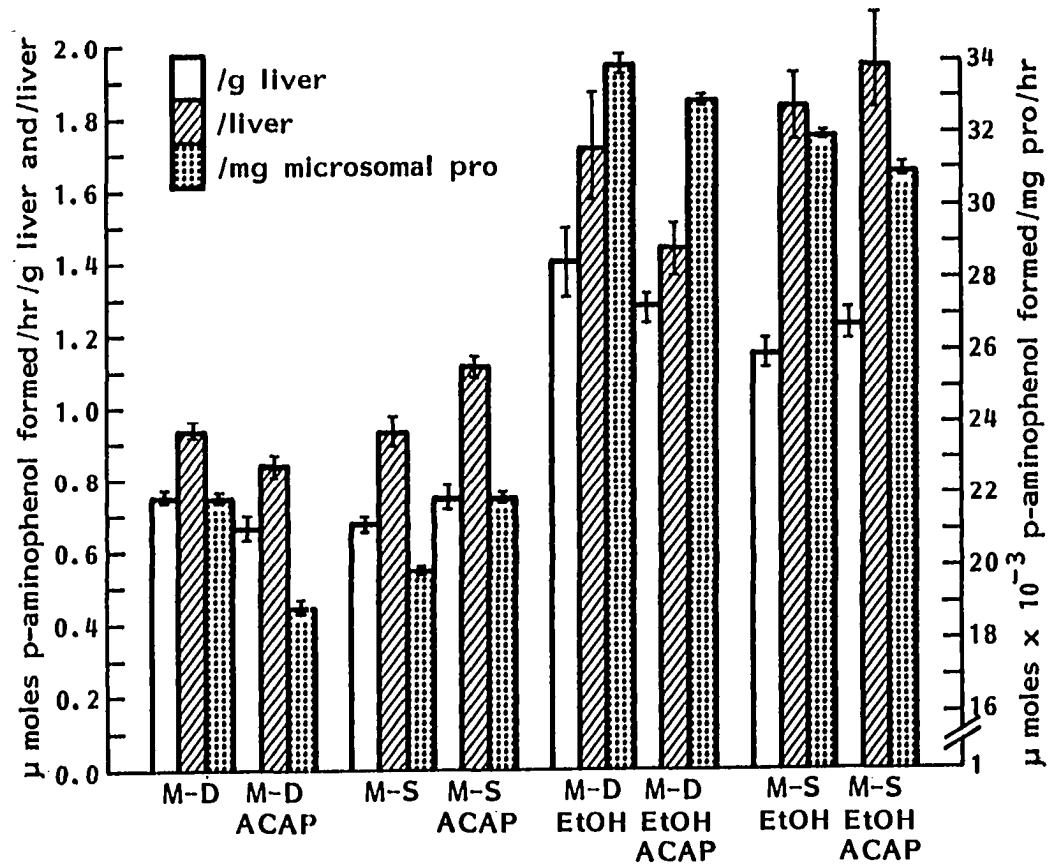


FIGURE 4. Relative liver weight (% BW)^a

^a $p < 0.01$ for EtOH.

ANILINE HYDROXYLASE ACTIVITY ^a



^ap < 0.0001 for EtOH

FIGURE 5. Aniline hydroxylase activity

and in the groups not consuming EtOH when expressed as change in absorbance per hour per gram of liver (Table 5).

Table 4. Serum inorganic sulfate concentration

Treatment	mM ^a	N
M-D	1.52 ± 0.16	5
M-D; ACAP	0.50 ± 0.34	5
M-S	1.31 ± 0.37	5
M-S; ACAP	0.85 ± 0.28	5
M-D; EtOH	2.32 ± 0.71	4
M-D; EtOH; ACAP	0.78 ± 0.38	3
M-S; EtOH	1.58 ± 0.16	5
M-S; EtOH; ACAP	1.23 ± 0.26	5

^a_p < 0.01 for ACAP.

Experiment 2

The extent of ¹⁴C-methyl-met, ³⁵S-met, and Na₂³⁵SO₄ incorporation into hepatic proteins is shown in Table 6,7 and 8 respectively. The amount of ¹⁴C-methyl-met incorporated represents the amount of met directly contributing to protein synthesis, while the amount of ³⁵S-met represents the amount of met directly involved in protein synthesis and the amount of met which is processed to donate its sulfur to cysteine

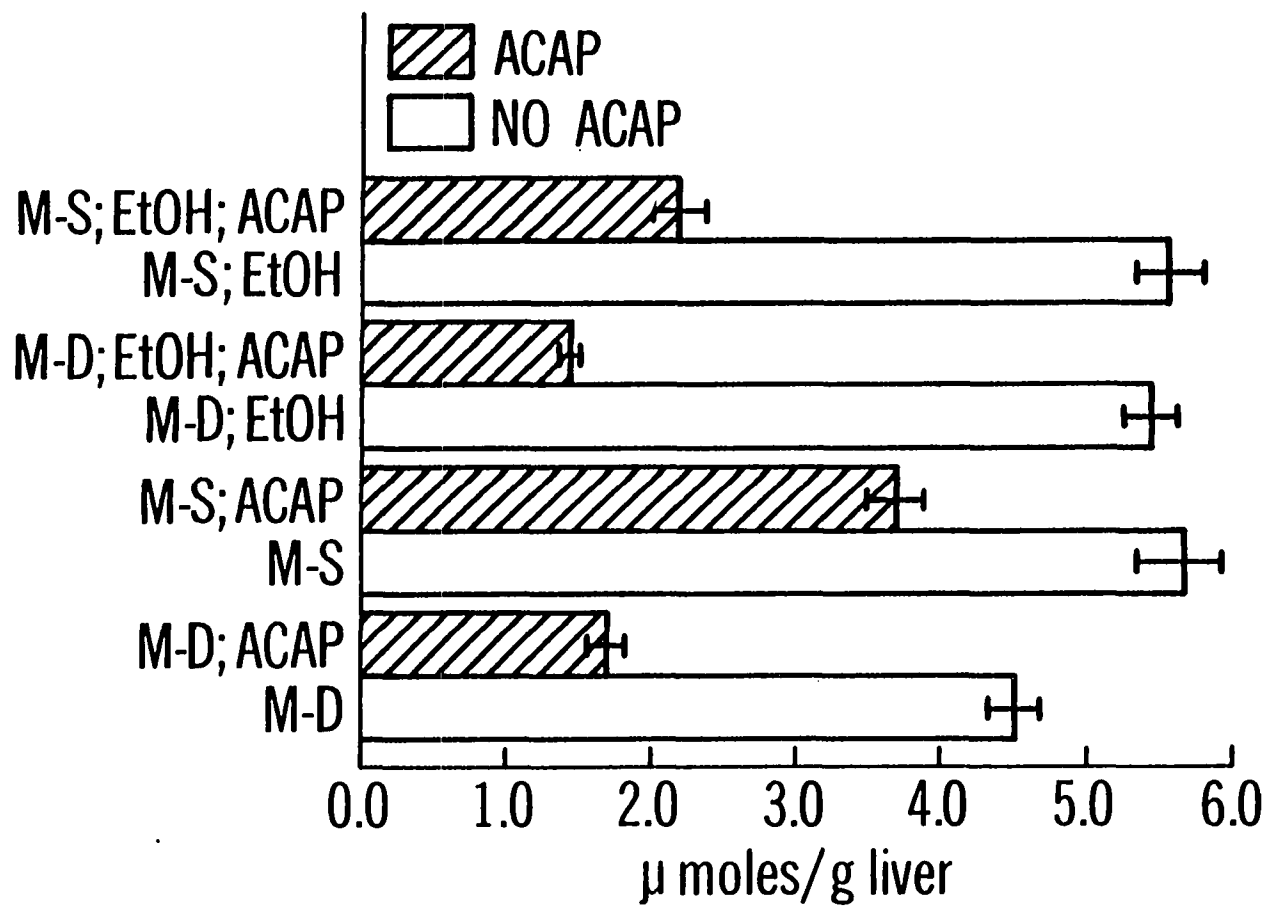


FIGURE 6. Hepatic GSH concentration^{abcd}

^ap < 0.01 for met.

^bp < 0.01 for ACAP.

^cp < 0.05 for met*ACAP.

^dp < 0.05 for EtOH*ACAP.

Table 5. Uridine-diphospho-glucuronosyl transferase activity 1 hr after 300 mg/kg i.p. ACAP

Treatment	(Δ Abs/hr)	/g liver ^a	/liver ^b	/mg microsomal pro ^c	N
M-D		3.28 \pm 0.13	4.23 \pm 0.27	0.11 \pm 0.01	6
M-D; ACAP		2.87 \pm 0.20	3.81 \pm 0.44	0.09 \pm 0.01	6
M-S		2.91 \pm 0.12	4.36 \pm 0.70	0.10 \pm 0.01	6
M-S; ACAP		2.99 \pm 0.20	4.50 \pm 0.86	0.10 \pm 0.01	6
M-D; EtOH		3.44 \pm 0.17	4.18 \pm 0.37	0.10 \pm 0.01	6
M-D; EtOH; ACAP		3.56 \pm 0.20	4.07 \pm 0.54	0.10 \pm 0.01	6
M-S; EtOH		3.70 \pm 0.27	6.08 \pm 0.69	0.11 \pm 0.01	6
M-S; EtOH; ACAP		3.69 \pm 0.25	5.94 \pm 0.73	0.10 \pm 0.01	6

^ap < 0.01 for EtOH.

^bp < 0.01 for met.

^cNo significant differences.

via the transsulfuration pathway, which then also can be incorporated into proteins. The amount of $\text{Na}_2^{35}\text{SO}_4$ incorporated may represent the amount of sulfate used for proteoglycan or other sulfate-requiring compound synthesis. After subtracting the amount of ^{14}C -methyl-met incorporation from the ^{35}S -met incorporation, the resulting amount could be a reflection of the amount of met that donates its sulfur to form cysteine or of the activity of the transsulfuration pathway. However, there was no consistent pattern of decreased transsulfuration pathway activity seen in the M-D groups from the data generated in Experiment 2. ^{14}C -methyl-met incorporation was decreased in the M-D groups and ^{35}S -met incorporation was decreased after ACAP administration.

Endogenous free hepatic met (Figure 7) was significantly increased in the EtOH-consuming groups and was reduced after ACAP administration. Endogenous met was used to calculate the extent of ^{35}C -methyl-met and ^{35}S -net incorporation while exogenous met was kept constant. There were no significant differences in hepatic endogenous free cysteine/cystine values (Table 9) as determined by automated amino acid analysis.

Experiment 3

Several animals from each group were used to determine dose and amount of radioactivity needed for later detection. The remaining animals were used for urine collection after a 300 mg/kg BW i.p. dose of ACAP (Table 10). ACAP-sulfate conjugate formation and free ACAP excretion was significantly increased in the M-S groups while ACAP-

Table 6. ^{14}C -methyl-methionine incorporation into PMS proteins

Treatment	% incorporation ^{a,b,c} ^{14}C radioactivity	nmoles ^{14}C me-met incorp per g liver ^{a,b}	nmoles ^{14}C -me-met incorp per mg pro/hr ^{b,c,d,e}
M-D	0.61 ± 0.07	0.623 ± 0.053	0.086 ± 0.006
M-D; ACAP	0.69 ± 0.10	0.691 ± 0.073	0.093 ± 0.010
M-S	1.04 ± 0.14	1.118 ± 0.239	0.165 ± 0.038
M-S; ACAP	0.72 ± 0.08	0.722 ± 0.095	0.099 ± 0.009
M-D; EtOH	0.53 ± 0.06	0.566 ± 0.046	0.084 ± 0.009
M-D; EtOH; ACAP	0.64 ± 0.07	0.613 ± 0.073	0.089 ± 0.012
M-S; EtOH	0.58 ± 0.06	0.605 ± 0.043	0.082 ± 0.007
M-S; EtOH; ACAP	0.61 ± 0.03	0.611 ± 0.055	0.079 ± 0.007

^a_p < 0.05 for met.

^b_p < 0.01 for EtOH.

^c_p < 0.05 for met*ACAP.

^d_p < 0.06 for met.

^e_p < 0.01 for met*EtOH.

Table 7. ³⁵S-methionine incorporation into PMS proteins

Treatment	% incorporation ^a ³⁵ S radioactivity	mmoles ³⁵ S-met incorp per g liver ^b	mmoles ³⁵ S-met incorp per mg pro/hr ^c
M-D	0.89 ± 0.09	0.957 ± 0.095	0.131 ± 0.011
M-D; ACAP	0.91 ± 0.09	0.956 ± 0.093	0.128 ± 0.014
M-S	0.98 ± 0.14	1.129 ± 0.145	0.163 ± 0.015
M-S; ACAP	0.92 ± 0.12	1.025 ± 0.152	0.143 ± 0.021
M-D; EtOH	0.86 ± 0.19	1.161 ± 0.187	0.169 ± 0.026
M-D; EtOH; ACAP	0.79 ± 0.06	0.818 ± 0.058	0.119 ± 0.010
M-S; EtOH	0.95 ± 0.08	1.121 ± 0.073	0.152 ± 0.011
M-S; EtOH; ACAP	0.86 ± 0.14	0.930 ± 0.142	0.121 ± 0.021

^aNo significant differences.

^bp < 0.08 for ACAP.

^cp < 0.05 for ACAP.

Table 8. $\text{Na}_2^{35}\text{SO}_4$ incorporation into PMS proteins

Treatment	% incorporation ^a ³⁵ S radioactivity
M-D	0.16 ± 0.03
M-D; ACAP	0.16 ± 0.04
M-S	0.21 ± 0.03
M-S; ACAP	0.24 ± 0.08
M-D; EtOH	0.21 ± 0.05
M-D; EtOH; ACAP	0.13 ± 0.03
M-S; EtOH	0.22 ± 0.07
M-S; EtOH; ACAP	0.23 ± 0.04

^a_p < 0.08 for met.

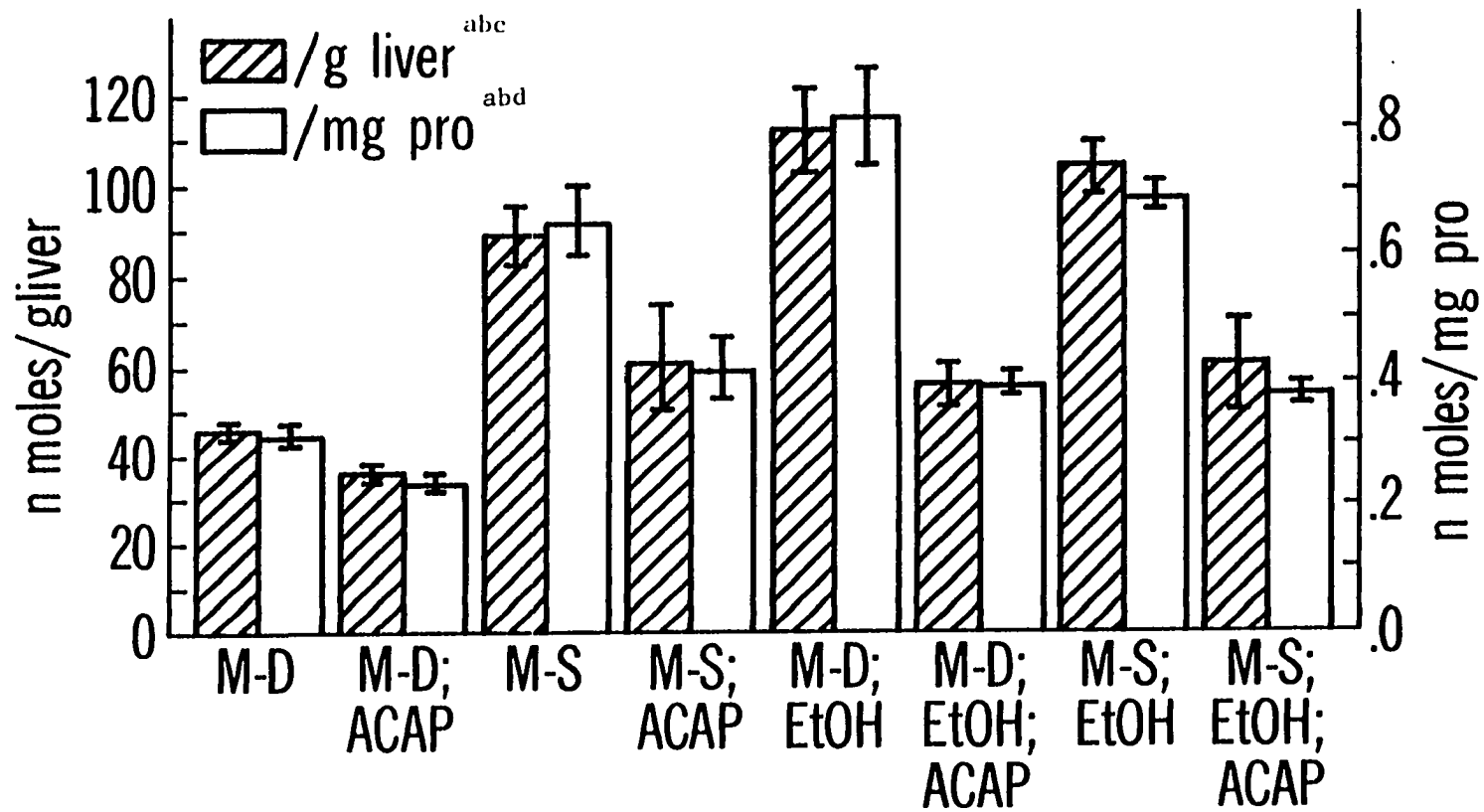


FIGURE 7. Hepatic free methionine

^ap < 0.01 for EtOH.

^bp < 0.01 for ACAP.

^cp < 0.05 for met*EtOH.

^dp < 0.01 for met*EtOH.

Table 9. Hepatic free cystine

Treatment	nmoles/g liver ^a	N
M-D	217.8 ± 29.8	5
M-D; ACAP	151.5 ± 24.0	5
M-S	137.4 ± 28.1	4
M-S; ACAP	178.3 ± 32.7	5
M-D; EtOH	191.4 ± 21.4	5
M-D; EtOH; ACAP	172.8 ± 5.4	5
M-S; EtOH	207.9 ± 54.0	5
M-S; EtOH; ACAP	147.5 ± 18.9	5

^aNo significant differences.

glucuronate formation was decreased in the M-S groups. There was greater ACAP-sulfate and ACAP-mercapturic acid conjugate formation in the EtOH consuming groups and less ACAP-glucuronate conjugate formation and less free ACAP excreted in the EtOH consuming groups.

Table 10. ACAP conjugates in 8-hr urine after 300 mg/kg BW i.p. ACAP

Treatment	ACAP-sulfate ^{a,b}	Percent distribution of recovered dose			N
		ACAP-glucuronide ^{a,b}	Free ACAP ^{a,b}	ACAP-mercapturate ^b	
M-D	11.58 ± 0.34	71.54 ± 1.19	2.45 ± 0.30	12.86 ± 0.84	9
M-S	14.77 ± 1.19	65.63 ± 2.37	3.66 ± 0.44	13.96 ± 1.06	10
M-D; EtOH	14.91 ± 0.83	64.14 ± 1.41	1.26 ± 0.16	17.38 ± 1.00	7
M-S; EtOH	16.67 ± 0.65	60.87 ± 1.07	1.97 ± 0.32	17.70 ± 0.37	10

^a_p < 0.01 for Met.

^b_p < 0.01 for EtOH.

DISCUSSION

Methionine-deficient mice and EtOH-consuming mice had a lower body weight than similar methionine-adequate control animals at the end of the pretreatment period even though pair-feeding kept caloric intake fairly constant. The M-D diet contained approximately 0.225% met which is 45% of the 0.5% met requirement. In young adult rats (360 gm), Seligson and Rotruck (2) showed weight loss after being fed a diet providing met at 0.2% of the diet for 28 days. Rats fed a diet containing 0.3% met gained weight but optimal weight gain occurred with met at 0.5% of the diet. Glazenburg et al. (1) also saw growth retardation as the level of dietary sulfur from met and cysteine was reduced in rats.

Hepatic microsomal changes have been seen in laboratory animals after chronic EtOH consumption. A proliferation of smooth endoplasmic reticulum is seen with a concomitant increase in the activity of some microsomal drug-metabolizing enzymes (22). Aniline hydroxylase activity (Figure 5) is clearly induced by EtOH consumption. The increased relative liver weight (Figure 4) is also good evidence for EtOH induction in EtOH consuming groups.

A reduction in serum inorganic sulfate after ACAP administration has been shown by others in rats and humans (25-27) and is also shown in Table 4. Serum inorganic sulfate concentration was decreased compared to control rats after rats were fed a diet which contained 75% of the met level in the control diet and increased after being fed a diet

containing 50% of the met in the control diet for 2 weeks (1). There was also no clear relationship found between dietary met and serum inorganic sulfate in the data in Table 4, suggesting that dietary sulfur amino acids were not the limiting factor for serum sulfate levels.

Hepatic GSH content was decreased as expected by met deficiency and as reported previously in mice and rats (1,2,28). GSH concentration was also predictably decreased after ACAP injections in animals fed both met levels, but to a greater extent in the M-D groups. The M-S mice should have a greater ability to protect the liver from ACAP induced necrosis.

The enzyme that catalyzes the conjugation reaction involving ACAP and uridine diphospho-glucuronic acid (UDPGA), UDPGT, showed an increase in activity per gm of liver in the EtOH consuming groups and a decrease in activity per liver in the methionine-deficient groups which may be attributed to a decrease in liver size. Magdalou et al.

(29) reported an increase in UDPGT activity in rats fed a balanced diet with only 48% of the sulfur containing amino acids of the control diet. Price and Jollow (30) have shown that UDPGT is not likely to be rate-limiting in the reaction measuring UDPGA using [³H]-diethylstilbestrol as a substrate and guinea pig liver microsomes as the source of UDPGT.

In the second experiment, radiolabelled met was incorporated into hepatic proteins in vitro to examine the activity of the transsulfuration pathway and how it is affected by treatment conditions. Finkelstein and Martin (31) followed the consumption of homocysteine

using rat liver homogenates from rats fed a standard chow diet. The remethylation enzymes, betaine-homocysteine methyltransferase (BH-MT) and methionine synthase or methyl-tetrahydrofolatehomocysteine methyltransferase (mTHF-MT) accounted for 53% of the homocysteine consumed while the cystathionine synthase reaction accounted for 46% of the homocysteine consumed. The percentage transsulfuration as seen by increases in cystathionine synthase catalysis rose to 89% after rats were fed a diet with excess protein, and was reduced by 83% in rats fed a protein deficient diet.

M-D mice would be expected to have less transsulfuration activity, while ACAP-treated mice would be expected to have greater transsulfuration activity because of the need for a rapid increase in cysteine concentration to serve as a substrate for GSH biosynthesis. However, an increase was not observed after calculating the amount of met that donates its sulfur to form cysteine from the incorporation data in Table 6, 7, and 8, as reflected in relative incorporation rates for ^{14}C -methyl-met, ^{35}S -met and $\text{Na}_2^{35}\text{SO}_4$ into PMS proteins. The incorporation of ^{14}C -methyl-met was decreased in the M-D groups which may indicate that an attempt was being made to conserve met for the transsulfuration pathway. The amount of ^{35}S -met being incorporated is less after ACAP which may indicate that the met is donating its sulfur to cysteine which is then conserved for GSH biosynthesis rather than for protein synthesis.

Hepatic met values (Figure 7) are in agreement with those of Finkelstein et al. (32). These authors found that by feeding rats an increasing amount of dietary protein, met content of liver increased. No significant difference occurred due to dietary met in the data in Figure 7; however hepatic met content was decreased after ACAP injections, suggesting that met was contributing to the cysteine synthesis and GSH for ACAP conjugation. However, this is not likely since hepatic cysteine/cystine levels were not changed after ACAP.

In experiment 3, the percent distribution of ACAP into sulfate conjugates was decreased in the M-D groups. There also was a greater percentage of sulfate and mercapturic acid conjugates formed in the EtOH consuming groups. Glazenburg et al. (1) reported a decrease in ACAP-sulfate when dietary met was lowered, but did not show a compensatory increase in ACAP-glucuronate as is seen in the data in Table 11. The effect of chronic EtOH consumption seems to be to increase ACAP conjugation by sulfate or GSH and to decrease the amounts converted to the glucuronate or excreted as free ACAP. EtOH increases the amount of ACAP converted to the toxic intermediate which may account for the increase in ACAP-mercapturic acid excretion. There is also a greater decrease in GSH concentration after ACAP in the EtOH consuming groups.

In summary, dietary met deficiency caused decreases in body weight, hepatic GSH content, ^{14}C -methyl-met incorporated into hepatic proteins and ACAP conjugated by sulfation. Ethanol consumption resulted in decreases in body weight and ^{14}C -methyl-met incorporated into hepatic

proteins, induction of hepatic drug-metabolizing enzymes, higher endogenous free hepatic met, and increased ACAP conjugated by sulfation and mercapturic acid formation. ACAP administration decreased serum inorganic sulfate concentration, hepatic GSH content, ³⁵S-met incorporation into hepatic proteins, and hepatic free methionine content. The interaction of EtOH consumption and met deficiency caused a larger decrease in protein in the PMS fraction of liver and an increase in the met content of the liver.

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SUMMARY AND DISCUSSION

By using the factorial design, the effects of dietary methionine deficiency and ethanol consumption on ACAP metabolism can be determined separately or together as possible interactive effects. Dietary methionine deficiency decreased hepatic glutathione concentrations and ACAP conjugation by sulfation, although serum inorganic sulfate concentration was not altered by dietary methionine content. Chronic ethanol consumption caused induction of the cytochrome P-450 dependent MFO system as shown by increased p-hydroxylation of aniline and increased relative liver weights. In the first paper, the p-nitroanisole O-demethylase activity was not increased by 15% ethanol in the drinking water. This may be because ethanol may specifically induce only the form of cytochrome P-450 (isozyme 3a) involved in aniline hydroxylation and ACAP metabolism (98,101). Ethanol ingestion also resulted in increased ACAP conjugated by GSH to form mercapturic acid. Hepatic GSH depletion, degree of covalent binding and mercapturic acid conjugate excretion after large ACAP doses correlate with hepatic necrosis but should only be used as indices of ACAP activation and not of hepatotoxicity.

Measures of hepatotoxicity used were PGOT activity increases and lethality determined by the LT_{50} . PGOT activity was greatly increased by the interaction of chronic ethanol consumption and methionine deficiency. The LT_{50} was reduced and PGOT was increased in the methionine deficient groups indicating that the extent of GSH depletion was greater presumably because of already diminished concentrations.

The effect of dietary methionine deficiency on the hepatic transsulfuration pathway activity could not be determined from the data generated from the in vitro protein synthesis experiment. The transsulfuration activity would be expected to be decreased (71) in methionine deficient states, resulting in decreased cysteine concentrations and therefore, depressed GSH levels and reduced capacity for GSH resynthesis after ACAP.

Optimal nutritional status for certain nutrients is required to maximize the ability of the animal to protect itself from ACAP induced hepatotoxicity. The chronic alcoholic may then be at an additional risk in taking ACAP because the dietary intake of high quality protein and other nutrients may be suboptimum.

The potentiation of ACAP-induced hepatotoxicity by ethanol is a current concern of the health care profession, especially since the chronic alcoholic may be more likely to choose ACAP over aspirin as an analgesic because it isn't associated with gastric bleeding or erosions. The reports on the increased hepatotoxicity seen in alcoholics (5,6,7) have led to suggestions to replace ACAP with the N-methyl substituted derivative which has not produced hepatic necrosis in animals (112). A more effective way to inform alcoholic consumers of the possible dangers of taking ACAP is also advocated. Interactive effects of EtOH and met deficiency were observed directly in PGOT elevation after ACAP, however indirect effects may also be present.

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