are hydroxylated in the α-methylene group by microsomal liver enzymes; mandelonitrile, for example, is produced as a metabolite of benzylcyanide [7, 14]. Benzaldehyde is then oxidized to benzoic acid which is conjugated with glycine to form hippuric acid. Cyanide, the other major urinary metabolite, is also partly detoxified to thiocyanate, a reaction catalysed by thiosulphate-cyanide-sulphur transferase (EC 2.1.1.1.) or rhodanese [17]. The excretion of CN^- and SCN^- together, corresponding to 86% of the dose, indicated that scission was the major metabolic pathway of mandelonitrile *in vivo*. The benzaldehyde formed concomitantly would be oxidized to benzoic acid and mainly converted to hippuric acid; 71.2% of the dose was excreted in this form. Conversion of mandelonitrile to mandelic acid occurred to the extent of 13.1% of the dose which constitutes a relatively minor but significant metabolic pathway. In Fig. 1, it is proposed that mandelonitrile is oxidized via an amide intermediate to mandelic acid, a reaction known to occur with other aromatic cyanides and nitriles [18]. Trace amounts of other metabolites detected by GLC but not characterized possibly include *p*-hydroxy-mandelic acid and phenylglyoxylic acid, which are known to be formed from the metabolism of mandelic acid in man [19].

The significant high levels of CN^- relative to SCN^- within the first 24 hr after dosing but not afterwards ($P < 0.05$) may indicate an initial saturation of the rhodanese enzyme, perhaps as a result of limitation of sulphur availability. This is in agreement with the pharmacokinetics of administered potassium cyanide in the rat [20] and dog [21]. Moreover, toxicity of laetrile to dogs occurred before CN^- could be converted to SCN^- [4]. Thus, the failure, rapidly and effectively to detoxify this initial large amount of CN^- formed from mandelonitrile accounts for the acute toxicity of this compound in the rat.

In summary, male and female Wistar rats were dosed orally with mandelonitrile (30 mg/kg), and metabolites were identified and estimated in urine. The cyanide plus thiocyanate excreted represented 86% of the dose, hippuric acid formed via the same metabolic pathway corresponded to 71% of the dose, while 13% of the dose was excreted as mandelic acid. A metabolic scheme was proposed for mandelonitrile (Fig. 1), involving a major toxication pathway yielding cyanide and a minor detoxication pathway to mandelic acid.

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Silymarin protection against hepatic lipid peroxidation induced by acute ethanol intoxication in the rat

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From the metabolic viewpoint, the liver is one of the organs primarily affected by ethanol consumption [1]. Some of the metabolic alterations induced by ethanol have been implicated in the production of liver damage after prolonged and excessive alcohol ingestion [1–4]. Among these, the hypothesis of ethanol-induced lipid peroxidative injury in the liver has been re-evaluated recently [5]. In experimental animals, it has been demonstrated that acute and chronic [5–8] ethanol administration produce a drastic increase in the hepatic content of reduced glutathione (GSH), the most important protective biomolecule against chemically-induced cytotoxicity [5, 9]. In fact, GSH can participate in the elimination of either reactive xenobiotics by conjugation [10], hydroperoxides by reduction [11], or free radicals by direct quenching [9]. Ethanol-induced liver GSH depletion has been observed concomitantly with an enhancement in hepatic lipid peroxidation, measured by different experimental procedures [5, 6, 12, 13]. Liver GSH depletion [14] and increased malondialdehyde levels [15] have also been reported in liver biopsy specimens from chronic alcoholic patients, when compared to basal values. These observations indicate that hepatic lipid peroxidation

is also a concomitant of alcohol abuse in man, which may conceivably be exacerbated by contributory factors such as microsomal enzyme induction, hepatic accumulation of iron [16], and/or nutritional deficiencies [5].

Recently, it was reported that (+)-cyanidanol-3 [(+)-Catechin], a flavonoid that has been used in the treatment of liver diseases [17], is able to reduce by 80% the liver GSH depletion induced by acute ethanol treatment and to abolish the lipid peroxidative response produced by the toxin [18]. This protective effect has been related to the antioxidant properties of the flavonoid [19, 20], which may inhibit the deleterious effect of the oxygen-related free radicals generated by the microsomal and cytosolic enzyme systems involved in ethanol and acetaldehyde oxidation in the liver [5, 18, 21]. Apart from (+)-cyanidanol-3, silymarin is another bioflavonoid that has been used as a therapeutic agent in many liver diseases [22–25]. It is extracted from the thistle *Silybum marianum* as a mixture of three structural isomers, silybin, silydianin and silychristin, the former being the most active component [26]. When given to experimental animals, the flavonoid protects the liver against hepatotoxic agents such as α-amanitin, phalloidin, galatosamine, carbon tetrachloride and thioacetamide [27–30]. Although the protective mechanism of silymarin has not been elucidated, it is generally accepted that the flavonoid exerts a membrane-stabilizing action preventing or inhibiting membrane peroxidation [31]. In fact, silymarin strongly inhibits peroxide formation induced by peroxidative agents both in liver mitochondria and microsomes [32]. In view of the antioxidant and cytoprotective properties described for silymarin, we studied the effect of a single intraperitoneal dose of the flavonoid on the changes in rat liver GSH levels and lipid peroxidation induced by acute ethanol intoxication.

Male Wistar rats (Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile) weighing 200–250 g were divided into four groups: group A received carboxymethyl cellulose (CMC)-glucose; group B, CMC-ethanol; group C, silymarin-glucose; and group D, silymarin-ethanol. A dose of 200 mg of silymarin/kg (Laboratorio Silesia, Santiago, Chile) as a 0.5% (w/v) suspension in 2% (w/v) CMC (4000 cp) was given intraperitoneally at time zero (5:00 p.m.), and the corresponding controls received an equivalent amount of 2% (w/v) CMC. The animals were fasted overnight, and 16 hr following silymarin treatment they were given either 5 g of ethanol/kg [as 30% (w/v) solution] or isocaloric amounts of glucose [as 40% (w/v) solution] by oral intubation. Studies were carried out after 6 hr of ethanol treatment in animals kept in a warm environment (25°). The administration of both silymarin and/or ethanol under these conditions did not result in significant changes in liver weight, liver weight/body weight ratio or hepatic protein content (Table 1). Glutathione levels were measured in 20 g/l liver homogenates prepared in a buffer solution containing 5 mM Tris (pH 7.4), 140 mM KCl and 1 mM EDTA, by the enzymatic assay described by Bernt and Bergmeyer [33]. GSH was measured with the methylglyoxal-glyoxalase I system at 240 nm, and oxidized glutathione (GSSG) was determined with the NADPH-glutathione reductase system at 340 nm. Liver lipid peroxidation was estimated by two different methods: the malondialdehyde thiobarbituric complex (MDA) and the evaluation of spontaneous chemiluminescence. The MDA measurement was performed according to Wilbur *et al.* [34] in a 1:4 liver homogenate prepared in 5 mM Tris (pH 7.4), 140 mM KCl and 1 mM EDTA, and malondialdehyde content was calculated by using $E = 1.56 \times 10^5$ litre · mole^{-1} cm^{-1} at 535 nm. Chemiluminescence was assessed in a Beckman LS-3150 P liquid scintillation counter in the out-of-coincidence mode, using 1:200 liver homogenates prepared in 5 mM Tris (pH 7.4), 140 mM KCl and 1 mM EDTA. Measurements were performed in 13 × 30 mm glass tubes introduced into standard 15-ml vials, previously equilibrated at 25° [20]. Backgrounds of the buffer alone (3 ml) were determined for each vial and were subtracted from the signals given by the liver homogenate. Results are expressed as counts per minute (cpm)/mg protein. Proteins were measured according to Lowry *et al.* [35]. All the reagents used were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Results are expressed as the mean ± S.E.M. The significance between mean values was assessed by Student's *t*-test for unpaired results.

Figure 1 shows the effect of acute ethanol administration on hepatic lipid peroxidation. As can be observed, ethanol treatment produced a marked increase in liver MDA formation (Fig. 1A) and in the spontaneous chemiluminescence (Fig. 1B). The thiobarbituric acid reaction with malondialdehyde is generally referred to as an indication of the secondary breakdown products of polyunsaturated fatty acids disrupted by free-radical oxidation [36]. In this process, low-level chemiluminescence is also produced from free radical interactions which can generate excited species [e. g. singlet oxygen ($^1O_2$) and excited carbonyl compounds $\diagdown\!\overset{*}{(C=O)}\!\diagup$] that on decay to ground state will emit photons [37]. Thus, both assays are complementary and allow a more general view of the lipid peroxidative process and are in agreement with earlier determinations of this phenomenon, assessed by the formation of diene conjugates [18], the chemiluminescence of the *in situ* rat liver [13], or by the biliary release of MDA in the anesthetized rat [16] after acute ethanol treatment. In this condition, a 36% reduction in the GSH content of the liver was observed, concomitantly with a 128% increase in GSSG levels, when animals given ethanol are compared to the respective controls (Table 2). These changes in GSH and GSSG levels represent a drastic decrease in the GSH/GSSG ratio (Table 2) of the liver as previously found [38].

The administration of silymarin to fed rats resulted in no changes in the basal lipid peroxidative rate of the liver (Fig. 1). However, when the flavonoid was given prior to acute ethanol treatment, it was able to completely suppress the increases in hepatic MDA formation and in spontaneous chemiluminescence observed when ethanol was given alone (Fig. 1). Possible mechanisms which may be responsible for the inhibition of ethanol-induced liver lipid peroxidation

Table 1. Reference parameters following silymarin pretreatment and ethanol intoxication in the rat*

	Body wt (g)	Liver wt (g)	g Liver/100 g Body wt	Hepatic protein content (mg/g liver)
CMC-Glucose (A)	219 ± 17	7.20 ± 0.62	3.28 ± 0.12	152 ± 16
CMC-Ethanol (B)	234 ± 12	7.95 ± 0.44	3.39 ± 0.08	145 ± 12
Silymarin-Glucose (C)	227 ± 16	7.15 ± 0.58	3.14 ± 0.19	158 ± 9
Silymarin-Ethanol (D)	231 ± 14	7.81 ± 0.24	3.38 ± 0.09	162 ± 17

* Results represent the mean ± S.E.M. for six rats. For all parameters, comparisons between the different experimental groups were not statistically significant.

Table 2. Effect of silymarin on the changes in liver glutathione levels induced by acute ethanol intoxication*

	GSH (μmoles/g liver)	GSSG (μmoles/g liver)	GSH_T (μmoles/g liver)	GSH/GSSG
CMC-Glucose (A)	6.40 ± 0.25	0.35 ± 0.15	7.20 ± 0.32	18.20 ± 1.72
CMC-Ethanol (B)	4.10 ± 0.40	0.80 ± 0.18	5.70 ± 0.48	5.12 ± 0.27
Silymarin-Glucose (C)	8.42 ± 0.52	0.48 ± 0.20	9.38 ± 0.65	17.54 ± 1.25
Silymarin-Ethanol (D)	6.75 ± 0.42	0.39 ± 0.22	7.53 ± 0.41	17.30 ± 1.11

* Data represent the mean ± S.E.M. for nine animals. Total GSH equivalents (GSH_T) were calculated according to the relation $GSH_T = GSH + 2GSSG$. Significance studies: GSH = A vs B and C ($P < 0.005$); A vs D (NS); B vs C and D ($P < 0.05$). GSSG = A vs B ($P < 0.005$); A vs C and D (NS); B vs C and D ($P < 0.01$). GSH_T = A vs B and C ($P < 0.005$); A vs D (NS); B vs C and D ($P < 0.01$). GSH/GSSG = A vs B ($P < 0.001$); A vs C and D (NS); B vs C and D ($P < 0.001$). NS = not significant.

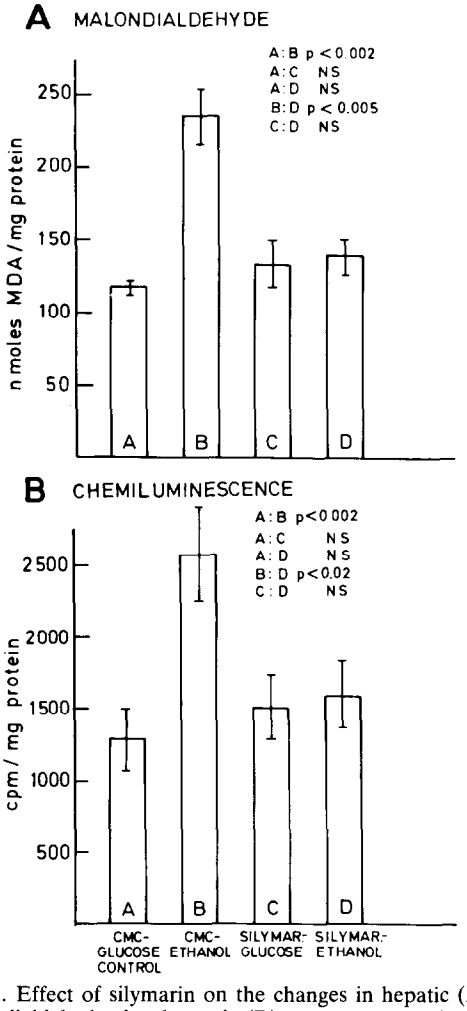

Fig. 1. Effect of silymarin on the changes in hepatic (A) malondialdehyde levels and (B) spontaneous chemiluminescence induced by acute ethanol intoxication. Each bar represents the mean ± S.E.M. for six rats in (A) and eight animals in (B). The P values shown were calculated by Student's *t*-test for unpaired data. NS = not significant.

by silymarin pretreatment include the following. (i) Silymarin by itself could act as a free radical scavenger intercepting those radicals involved in ethanol metabolism by microsomal enzymes [21, 39]. Thus, by trapping oxygen-related free radicals, silymarin could hinder their interaction with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes leading to MDA formation and light emision (Fig. 1). In this respect, silymarin would share with (+)-cyanidanol-3 [19, 20], an antioxidant mechanism of action acting as free radical scavengers. (ii) Silymarin pretreatment exhibited a novel effect on the glutathione status of the liver cell (Table 2), not observed in experiments using (+)-cyanidanol-3 [18]. As can be observed in Table 2, silymarin significantly increased the hepatic content of both GSH and GSSG, thus enhancing by 32% the content of total GSH equivalents (GSH + 2GSSG) of the liver, when compared to control rats given CMC. It is important to point out that this effect of silymarin administration did not alter the glutathione redox state of the liver, as the GSH/GSSG ratio was not modified by the treatment (Table 2). Silymarin-induced enhancement in total GSH equivalents expressed per g of liver (Table 2) was also observed when values are referred to body weight (Control CMC: 267.0 ± 25.3 (6) μmoles/kg body weight; Silymarin: 392.0 ± 14.1 (6); 46% increase; $P < 0.005$) or to hepatic protein content (Control CMC: 47.3 ± 4.0 (6) nmoles/mg protein; Silymarin: 60.1 ± 3.2 (6); 27% increase; $P < 0.005$) due to the lack of effect of the flavonoid on these reference parameters (Table 1). These results suggest that a higher content of glutathione in the liver, in conditions in which its redox state is kept constant, would afford the tissue a better protection against an oxidative stress, thus contributing to the abolishment of ethanol-induced lipid peroxidation (Fig. 1). This is supported by the lack of changes in the GSH/GSSG ratio following ethanol administration in silymarin-pretreated rats compared to the drastic decrease (68%) found when ethanol was given alone (Table 2). The mechanism of silymarin-induced enhancement of hepatic glutathione content is currently under study in our laboratory.

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The inhibition of bovine liver dihydrofolate reductase by tricyclic antidepressant drugs

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The inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate NADP oxidoreductase EC 1.5.1.3) by folic acid analogues has been well documented [1, 2]. The tricyclic anti-depressant drugs possess little structural similarity to these folate-analogue inhibitors and would not be expected to inhibit dihydrofolate reductase. The present study, however, reports significant inhibition of the bovine liver enzyme by such drugs. Six tricyclic drugs were studied; amitriptyline, butriptyline, desmethylnortriptyline, imipramine, nortriptyline and protriptyline.

Materials and methods

Materials. Folic acid and bovine liver dihydrofolate reductase were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Concentrated hydrochloric acid was from Fisons Scientific Apparatus (Loughborough, Leicestershire). Reduced NADP was obtained from Cambrian Chemicals (Croydon, U.K.). All other chemicals were from British Drug Houses Chemicals Ltd. (Poole, Dorset, U.K.). All reagents were of the highest grade or purity available.

Synthesis of dihydrofolate. The method employed was that of Wilmanns [3] and involved reduction of folic acid in the presence of ascorbic acid and sodium dithionite. The unstable dihydrofolate was synthesised prior to each experiment. The crystals obtained were suspended in 5 mM hydrochloric acid and kept in the dark at 0°. Concentrations of dihydrofolate were determined using the published molar absorbance coefficient of a solution of dihydrofolate in 10 mM potassium hydroxide ($= 22 \times 10^3$ l/mole/cm at 283 nm).

Enzyme assays. The method used was based on that of Wilmanns [3]. In a final reaction volume of 0.5 ml the following components were present: citrate buffer (0.1 M pH 5.4), 2-mercaptoethanol (12.5 mM), NADPH (varied in five stages from 0.04 to 1 mM), dihydrofolate (DHF) (varied in five stages from 1.4 to 34.7 mM) and dihydrofolate reductase (13 mIU/ml). Where appropriate, the tricyclic drug under investigation was present in the final reaction mixture at four concentration levels in the range of 4–200 μM. In a control experiment the inhibition of the enzyme by methotrexate (amethopterin, 4-amino-