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Hymenoxon: biologic and toxic effects

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Hymenoxys odorata (bitterweed) is an economically important poisonous range plant indigenous to the southwestern United States [1]. Hymenoxon, a sesquiterpene lactone, has been isolated from bitterweed, and laboratory studies have demonstrated that this compound elicits toxic symptoms in goats, rabbits and sheep which resemble those reported for bitterweed poisoning in the field [2, 3]. Previous studies on the mechanism of action of hymenoxon have been equivocal [4–12]. For example, pretreatment of mice with carbon tetrachloride, a compound which partially inactivates hepatic microsomal monooxygenases, affords some protection from the acute toxicity of this compound [9]. However, pretreatment of mice with either phenobarbital or Aroclor 1254, two inducers of hepatic monooxygenases and drug-metabolizing enzymes, does not increase the toxicity of hymenoxon [9]. Kim and coworkers have also reported that the antioxidant ethoxyquin (EQ) [10] and cysteine [11] offer some protection from the toxicity of hymenoxon in sheep and mice. In contrast, butylated hydroxyanisole (BHA) increases the LD_{50} of hymenoxon in mice but not sheep [10]. Since BHA and EQ induce hepatic glutathione transferases, it is possible that alterations in hepatic thiol levels and their conjugating enzymes may be important determinants in the mechanism of action of hymenoxon [13, 14]. However, BHA and EQ also act as cellular radical scavengers and induce microsomal cytochrome P-450-dependent monooxygenases, and these processes may also be involved in hymenoxon toxicity [15].

This study probes the mechanism of action of hymenoxon by determining the effects of a toxic dose of this compound (20 mg/kg) on mouse hepatic drug-metabolizing enzymes and related activities, including the microsomal monooxygenases, cytochrome P-450 levels, relative hepatic levels of reduced and oxidized glutathione (GSH and GSSG, respectively), GSH *S*-transferase (GSH Tase), GSSG reductase (GSSG Red), GSH peroxidase (GSH Px) and lipid peroxidation (LP) (determined as malondialdehyde levels in liver tissue).

Materials and methods

Hymenoxon (m.p. 135.5–136.5°, molecular weight 282.3) was extracted from dried ground bitterweed by the procedure of Kim *et al.* [3]. Corn oil/dimethyl sulfoxide (DMSO) (10 ml/kg) and hymenoxon (20 mg/kg) in corn oil/DMSO) were administered i.p. to immature male ICR mice (25–30 g, four mice for each time point) as a single dose 4, 8, 12, 72, 192 and 240 hr before the animals were killed. The mice were maintained on food and water *ad lib.* and were fasted 12 hr before sacrifice. Mice were killed by cervical dislocation, and the livers were immediately perfused with cold isotonic saline, pH 7.3.The livers were excised, weighed and minced in 0.25 M sucrose, pH 7.4. The microsomal (100,000 *g*) and soluble (supernatant) fractions were isolated by differential centrifugation as described by Mazel [16] and stored at 10°. Biochemical determinations were performed on the fresh preparations.

The cytochrome P-450 content was determined spectrophotometrically by difference spectrum using the molar extinction coefficient of 91 mM^{-1} cm^{-1} [17]. Dimethylaminoantipyrine (DMAP) *N*-demethylase and arylhydrocarbon hydroxylase (AHH) were measured as previously described [18]. GSH Tase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically at 340 nm [19]. GSH Px activity towards H_2O_2 and GSSG Red activity were assayed spectrophotometrically by determining the rate of NADPH oxidation at 340 nm, using the molar extinction coefficient for NADPH of 6.22×10^6 M^{-1} cm^{-1} [20]. The extent of microsomal lipoperoxidation was determined by measuring malondialdehyde production as thiobarbituric acid (TBA)-reactive substances at 535 nm [21]. The protein content of microsomal and soluble fractions was determined by the method of Lowry *et al.* [22]. Level of GSH and GSSG were measured in a tissue homogenate using an enzymatic recycling procedure [23]. All biochemical determinations were measured in fasted mice. Hymenoxon was added to the *in vitro* incubation of different compounds varying in sulfhydryl content. Hymenoxon (0.75 mg) in 0.1 ml of DMSO was added to 0.9 ml of 0.25 M phosphate buffer, pH 6.5, containing the polypeptide (3.0 mg). The solution was incubated at 20° for 30 min, and the reaction was terminated by the addition of ethyl acetate (400 μl) which was used to extract the unreacted hymenoxon. The amount of hymenoxon reacting with the polypeptides was derived from the quantitation of the unreacted compound using the appropriate standard curves determined for recoveries of hymenoxon employing a Tracor 565 gas chromatograph equipped with a flame ionization detector containing a 3 ft column packed with 3% OV-1 on Chromasorb W. The

carrier gas was nitrogen at a flow rate of 35 ml/min. The injector temperature was 150°, and the column temperature was programmed to 300° at 10°/min. Benzo[*a*]pyrene (B[*a*]P), NADP, glucose-6-phosphate, NADPH, glucose-6-phosphate dehydrogenase, GSSG Red, GSH and GSSG were purchased from the Sigma Chemical Co., DMAP and CDNB were purchased from the Aldrich Chemical Co., and carbon monoxide was purchased from the Matheson Chemical Co. Tritiated B[*a*]P (20 Ci/nmole) was purchased from the Amersham Corp. The statistical significance between the means of the data obtained from the hymenoxon-pretreated animals compared to the corn-oil-treated controls was determined using the method described by Dunnett [24].

Results and discussion

Antioxidants afford protection from the toxic effects of several compounds including hymenoxon, monocrotaline, aflatoxin B-1 and carbon tetrachloride [10–12, 25–27]. The latter toxic chemical is metabolically activated in the liver by cytochrome P-450-dependent monooxygenases to form a trichloromethyl radical which alkylates cellular proteins (including cytochrome P-450) and other macromolecules and initiates lipoperoxidation [27]. Not surprisingly, antioxidants, cysteine and glutathione which act as radical scavengers can protect from this type of cellular damage. The data summarized in Table 1 clearly differentiate between the mechanism of the hepatotoxicity of hymenoxon and carbon tetrachloride. Pretreatment of the mice with a hepatotoxic dose of hymenoxon (20 mg/kg) did not significantly alter activities of the hepatic microsomal monooxygenases, DMAP *N*-demethylase or AHH, or hepatic malondialdehyde formation. In contrast, treatment of rodents with carbon tetrachloride results in a decrease in hepatic cytochrome P-450 and related enzyme activities and an increase in malondialdehyde formation and hepatic lipoperoxidation. These results suggest that antioxidants do not protect mice from hymenoxon toxicity by a radical trap mechanism. Ethoxyquin and butylated hydroxyanisole also modulate the toxicity of many chemicals by their activities as inducers of glutathione *S*-transferase which can lead to an increase in the detoxication of alkylating agents by enhancing the formation of glutathione conjugates [14, 15, 25]. The effects of hymenoxon on cellular glutathione levels and related enzyme activities are summarized in Table 2. For up to 240 hr after treatment with a toxic dose of hymenoxon (20 mg/kg), the hepatic levels of glutathione (reduced and oxidized) and the activities of glutathione *S*-transferases, glutathione reductase and glutathione peroxidase were not significantly different from the values obtained for control (untreated) mice (Note: the hepatic levels of GSH were lower than values normally reported and this may be attributed in part to the fasting [28].)

The inability of hymenoxon to decrease hepatic levels of GSH and GSSG when viewed in conjunction with the antagonistic effect of L-cysteine on toxicity [11] presents a somewhat paradoxical situation. Hymenoxon is known to react with tissue sulfhydryls and has been shown to inhibit phosphofructokinase *in vitro* [12]. Lactones in general exert a cytotoxicological effect on tumors and this has been attributed to their action with sulfhydryl groups of this enzyme [29]. This is supported by comparing the LD_{50} values for hymenoxon ($LD_{50} = 16.2$ mg/kg) and hymenolane ($LD_{50} > 200$ mg/kg) [6]. The more toxic compound, hymenoxon, contains a highly reactive α, β-unsaturated carbonyl functionality whereas this group is not present in hymenolane (Fig. 1). Figure 2 illustrates the *in vitro* reactivity of hymenoxon with polypeptides which differ in their free thiol content, and it is evident that reactivity increases with increasing thiol content. Our failure to detect any reduction in cellular GSH levels may be due to the lack of specificity of the method used for determining GSH and GSSG. Current studies in our laboratory are focused on the development of more specific methods for GSH analysis, the effects of GSH and cysteine on the toxicity of hymenoxon and related sesquiterpene lactones, and the identification of *in vivo* and *in vitro* adducts of hymenoxon with cysteine-containing amino acids and polypeptides.

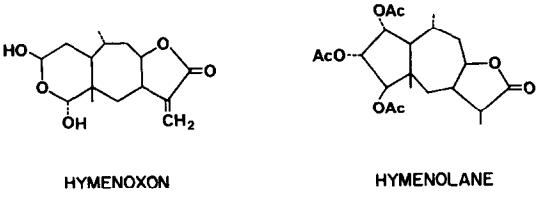

Fig. 1. Structures of hymenoxon and hymenolane. (Adapted from Ref. 6).

Table 1. Effect of hymenoxon on mouse hepatic cytochrome P-450 concentration, aryl hydrocarbon hydroxylase (AHH) and DMAP *N*-demethylase activity*

Hours post-treatment	Hymenoxon treatment	Cytochrome P-450 (nmoles/mg protein)	AHH† (pmoles/min/mg protein)	DMAP *N*-demethylase‡ (nmoles CHO/min/mg protein)
4	−	0.304 ± 0.084	12.35 ± 8.00	2.57 ± 0.80
	+	0.259 ± 0.047	23.25 ± 5.96	2.24 ± 0.26
8	−	0.248 ± 0.045	14.72 ± 5.14	2.15 ± 0.70
	+	0.194 ± 0.045	9.12 ± 3.20	1.94 ± 0.17
12	−	0.211 ± 0.084	10.56 ± 6.64	1.83 ± 0.24
	+	0.261 ± 0.057	18.27 ± 8.78	2.37 ± 0.78
72	−	0.227 ± 0.039	11.57 ± 8.92	2.73 ± 0.75
	+	0.234 ± 0.033	12.61 ± 8.00	4.49 ± 1.17
192	−	0.284 ± 0.025	10.99 ± 3.82	2.53 ± 0.53
	+	0.286 ± 0.025	7.35 ± 1.48	2.25 ± 0.49
240	−	0.238 ± 0.072	11.19 ± 2.31	2.60 ± 0.40
	+	0.285 ± 0.071	12.39 ± 3.18	3.01 ± 0.39

* All values are not significantly different ($P < 0.05$) from the control.
† Substrate: benzo[*a*]pyrene.
‡ Substrate: 4-dimethylaminoantipyrine.

Table 2. Effect of hymenoxon on enzymes related to glutathione (GSH) metabolism and microsomal lipid peroxidation (LP) in mouse liver*

Hours post-treatment	Hymenoxon treatment	GSH Tase† (μmoles/mg/in)	GSH Px† (nmoles/min/mg)	GSSG Red† (nmoles/min/mg)	LP†(nmoles TBA-reactive products/mg protein/10 min)	GSSG (μmoles/g liver)	GSH (μmoles/g liver)	GSSG and GSH (μmoles/g liver)
4	−	1.84 ± 0.48	0.17 ± 0.06	0.03 ± 0.00	2.62 ± 0.28	0.18 ± 0.10	3.60 ± 1.28	3.79 ± 1.19
	+	2.15 ± 0.30	0.27 ± 0.04	0.04 ± 0.02	2.78 ± 0.38	0.25 ± 0.19	2.18 ± 0.54	2.44 ± 0.47
8	−	2.72 ± 0.86	0.25 ± 0.03	0.06 ± 0.00	2.59 ± 0.20	0.08 ± 0.15	2.27 ± 0.89	2.34 ± 0.85
	+	1.89 ± 0.61	0.18 ± 0.02	0.06 ± 0.00	2.47 ± 0.18	0.24 ± 0.17	1.55 ± 0.06	1.79 ± 0.55
12	−	1.60 ± 0.52	0.19 ± 0.02	0.05 ± 0.02	2.38 ± 0.17	0.46 ± 0.07	1.43 ± 0.68	1.89 ± 0.73
	+	2.15 ± 0.68	0.91 ± 0.03	0.05 ± 0.01	2.62 ± 0.21	0.50 ± 0.49	1.17 ± 0.74	1.67 ± 0.71
72	−	2.54 ± 0.63	0.22 ± 0.03	0.05 ± 0.01	2.79 ± 0.40	0.26 ± 0.16	1.91 ± 0.93	2.17 ± 1.08
	+	2.37 ± 0.73	0.19 ± 0.03	0.04 ± 0.01	2.57 ± 0.25	0.26 ± 0.13	1.21 ± 1.21	1.49 ± 1.28

* All values are not significantly different ($P < 0.05$) from the control.
† Also unchanged at 192 and 240 hr post-treatment.

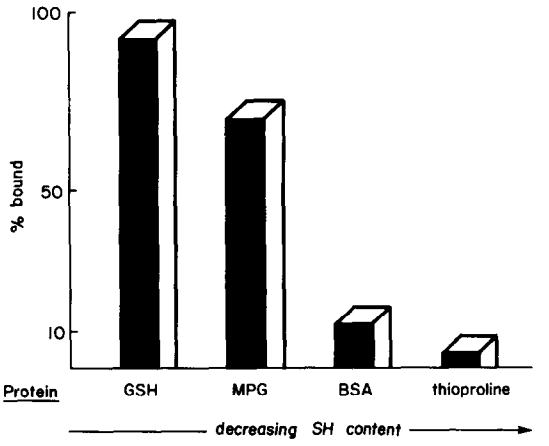

Fig. 2. Capacity of glutathione (GSH), *N*-2-mercaptopropionyl glycine (MPG), bovine serum albumin (BSA) and thioproline to bind hymenoxon *in vitro*.

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Inactivation of catalase with 3-amino-1,2,4-triazole: an indirect irreversible mechanism

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The metabolism of oxygen in biological environments generates a number of reactive chemical species which, if not controlled, can cause severe localized injury to the tissue [1]. Of these reactive oxygen metabolites, it is probably hydrogen peroxide which is the most toxic since it is able to oxidize proteins directly or can act as a precursor to produce the extremely reactive hydroxyl radical [2]. It is therefore not surprising that the hydroperoxidases, particularly catalases, are considered to play a pivotal role in protecting many tissues from the toxic effects of hydrogen peroxide [3].

Elucidation of the biological role of catalase has been limited by the absence of specific pharmacological inhibitors. While 3-amino-1,2,4-triazole is a compound which has been used to inhibit catalase in a number of studies [4–6], there is, in fact, some evidence to suggest that it does not have a direct effect upon catalase [7]. Nevertheless, systemic administration of 3-amino-1,2,4-triazole has been shown to result in inhibited hepatic, renal and ocular catalase activity [4–6]. *In vivo* experiments with 3-amino-1,2,4-triazole have provided the basis for the suggestion that catalase in the ocular tissues may fulfill a role in the maintenance of lens transparency [5, 6]. In contrast, the use of 3-amino-1,2,4-triazole in short term, *in vitro*, studies with ocular tissues has proved to be ineffective [8].

In this study, we have further characterized the mechanism of action of 3-amino-1,2,4-triazole upon purified catalase and the catalase activity of rabbit liver and rabbit iris-ciliary body. Our findings suggest that 3-amino-1,2,4-triazole has little direct influence upon catalase but is able to irreversibly inactivate a significant amount of catalase activity via an indirect mechanism.

Methods and materials

Chemicals. 3-Amino-1,2,4-triazole was purchased from the Sigma Chemical Co. (St. Louis, MO). Purified bovine liver catalase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Hydrogen peroxide was purchased as a 30% analar grade solution from the Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were reagent grade unless otherwise stated. Tissue incubations were undertaken in modified Tyrode's solution which contained 145 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 5.5 mM dextrose at a pH of 7.4.

Measurement of catalase activity. Catalase causes the breakdown of hydrogen peroxide to water and oxygen according to the overall reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The liberation of oxygen can be monitored polarographically using an oxygen electrode, enabling a straightforward method of measurement to determine catalytic activity in biological samples [9, 10]. This method was employed in the present study using a Clark type oxygen electrode (Yellow Springs, CA): 2 ml of potassium phosphate buffer (50 mM, pH 6.0) containing 10^{-3} M hydrogen peroxide was placed in a continuously stirred constant temperature (25°) chamber containing the oxygen electrode. The release of oxygen following introduction of the purified catalase or tissue homogenates (50 μl volumes) was recorded graphically on a chart recorder and used to compute the rate of breakdown of hydrogen peroxide.

Tissue preparation. The tissues (liver and iris-ciliary body) were obtained from albino rabbits (2–3 kg) which were killed painlessly by an overdose of sodium pentobarbital. The liver was excised and a portion washed in ice-cold Tyrode's solution to remove excess blood. The iris-ciliary body was carefully dissected from the enucleated eyes as described in detail previously [11]. The tissues were gently blotted dry on filter paper, weighed, and then thoroughly homogenized in ice-cold Tyrode's solution. Since the liver has considerable catalase activity, less concentrated homogenates of liver tissue were prepared (25 mg wet weight/ml Tyrode's) relative to the iris-ciliary body homogenates (50 mg wet weight/ml Tryode's).

Incubations with 3-amino-1,2,4-triazole. (1) Purified catalase. Commercially obtained catalase was diluted with Tyrode's solution to a final concentration of 325 units/ml. The diluted solution of catalase was then incubated for 4 hr at 37° in either the absence or the presence of 3-amino-1,2,4-triazole (10–100 μg/ml). At given periods of time (5 min and 4 hr) during the incubation, the catalase activity was determined polarographically as described above.

It has been suggested that 3-amino-1,2,4-triazole might inhibit catalase in the presence of hydrogen peroxide [7]. To examine this issue, additional experiments were undertaken in which 3-amino-1,2,4-triazole was incubated with catalase in the presence of hydrogen peroxide. In these experiments, catalase (162.5 units/ml) was incubated with 3-amino-1-2-4-triazole (100 μg/ml) for 4 hr at 37° in phosphate buffer containing 10^{-3} M hydrogen peroxide. The final concentration of catalase was less than used in the experiments described above to ensure that the hydrogen