and total absorbance of the original radish juice, were more striking, and *N*-nitrosodimethylamine formation was inhibited by 56% at pH 3.0.

DISCUSSION

Japanese radish (*R. sativus*) is one of the most common vegetables in Japan, and its juice lost more nitrite than other vegetable juices. While the major nitrite-reacting components in the radish juice have not yet been identified, it may be labile phenolic-like substances judging from strong adsorption to anion exchangers, characteristic bathochromic shift of UV-absorption spectrum at an alkaline pH, and coloration by chloroimide reagent. In other sorts of radish, phenolic substances such as *p*-coumaric acid, caffeic acid, and ferulic acid have been found by extraction under rather severe conditions (Stohr and Herrmann, 1975). UV-absorption spectra of the unstable substances in Japanese radish obtained in the present study were not identical with those of any commonly known plant phenolics.

Nitrite is produced under variety conditions: by salivary (Ishiwata et al., 1975; Speigelhadler et al., 1976; Tannenbaum et al., 1976) and by bacterial (Ayanaba and Alexander, 1973) reduction of nitrate present in vegetables and endogenously by nitrifying bacteria in the body (Tannenbaum et al., 1978), which is then available for carcinogenic nitrosamine formation under gastric conditions. Ascorbic acid (Mirvish et al., 1972; Fan and Tannenbaum, 1973) and phenolics (Gray and Dugan, 1975; Yamada et al., 1978), which distribute in vegetables, have been shown to reduce nitrite and thus inhibit the nitrosamine formation, although the latter have contradictory effects depending upon the conditions (Challis and Bartlett, 1975; Walker et al., 1975; Davies and McWeeny, 1977; Kurechi et al., 1979). Japanese radish juice inhibited nitrosamine formation by reaction between nitrite and secondary amines under acidic conditions. This inhibition of nitrosamine formation may be due to the nitrite-reacting phenolic substances rich in the juice rather than to ascorbic acid.

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Received for review February 27, 1980. Accepted August 6, 1980.

Tissue Distribution and Disposition of Hymenoxon

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Hymenoxon reacts with endogenous constituents of blood, but the sulfhydryl group is not the primary reactive site. Hymenoxon is excreted as glucuronides via urine and bile.

Hymenoxys odorata bitterweed), *Helenium hoopsii* (sneezeweed), and *Baileya multiradiata* (Desert baileya) are toxic range plants that belong to the Composite family. These plants are experimentally poisonous to sheep, goats, and rabbits, but field cases of poisoning are observed mainly in sheep. Sheep intoxicated with bitterweed, sneezeweed or baileya show signs of anorexia, emaciation, depression, vomition, and muscle tremors. In some cases of bitterweed poisoning, convulsions with opisthotonus have been observed (Sperry et al., 1964; Rowe et al., 1973; Lewis and Dollahite, 1960; Kingsbury, 1964).

Hymenoxon, a toxic sequiterpene lactone (Figure 1), has been isolated and characterized from bitterweed by Kim et al. (1974, 1975) and Ivie et al. (1975). Hymenoxon has also been identified in seenzeweed (Hill et al., 1977; Ivie et al., 1976) and baileya (Hill et al., 1977). The ip LD_{50} of the crystalline compound in sheep is ca. 7 mg/kg. The iv LD_{50} and LD_{90} of partially purified preparations of the lactone for the dog are 30 and 50 mg/kg, respectively. Hymenoxon intoxication in dogs receiving an acute iv in-

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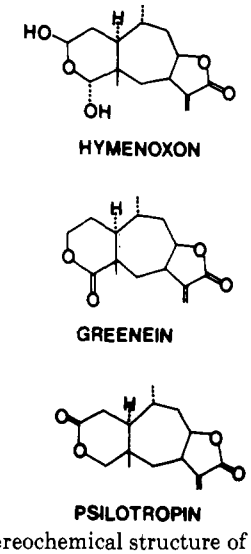

Figure 1. The stereochemical structure of three sesquiterpene lactones.

jection of the partially purified lactone produced tonic-clonic convulsions which were aggravated by external stimuli. Profuse salivation and vomition also occurred in the dog. The typical signs of depression and convulsions suggest that hymenoxon affects the CNS (Kim et al., 1974).

The biochemical fate of sesquiterpene lactones has not been studied in animal systems; therefore, the biotransformation products of these compounds have not been elucidated. Compounds that contain an α,β-unsaturated carbonyl functional group are known to react with the sulfhydryl group of glutathione. The glutathione portion of the complex is converted to *N*-acetylcysteine, and the compound is excreted as the mercaptouric acid (Kaye, 1973; Boyland and Chasseaud, 1970; Clapp and Young, 1970; Chasseaud, (1973). Compounds containing hydroxy groups can be conjugated with glucuronic acid or the sulfate group (La Du et al., 1971) in mammalian systems. The purpose of this study was to determine the distribution of hymenoxon in the rabbit after oral and iv adminstration and to determine the biotransformation products of hymenoxon.

EXPERIMENTAL SECTION

Chemicals. Hymenoxon (mp 135–136 °C; reported mp 135–142 °C) was extracted from bitterweed and repeatedly recrystallized by the procedure of Kim et al. (1975). Tritiated hymenoxon was prepared by New England Nuclear by reacting 100 mg of hymenoxon in dimethyl sulfoxide $Me_2(SO)$ with 10 Ci of tritiated water 5% Rh/Al_2O_2 as a catalyst. The tritiated hymenoxon (193 mCi/100 mg in 10 mL of Me_2SO) was purified on silica gel preparative thin-layer plates using a solvent system of chloroform–acetone (7:3). The [^{3}H]hymenoxon was eluted from the silica gel with ethyl acetate. The activity of the purified hymenoxon was 1.04 mCi/mL.

Instrumentation. The tritium content of animal tissues was oxidized to water and distilled into glass scintillation vials containing Monophase 40 scintillation cocktail in a Model 306 Tri-Carb sample oxidizer (Packard Instruments). The tritium content of each vial was measured in a Model 3330 Tri-Carb scintillation spectrometer (Packard Instruments).

A Varian 2100 or a Barber-Colman 5000 gas chromatograph was employed to analyze hymenoxon in the various biological fluids. Each instrument was equipped with a flame ionization detector.

Reaction of Hymenoxon with Blood. Whole blood was collected from the jugular vein of sheep with a syringe coated with heparin, and the blood was stored under nitrogen in a septum-sealed bottle. The blood was used the day of collection and was kept under nitrogen during all procedures to prevent the oxidation of sulfhydryl groups.

The sulfhydryl content of whole blood, washed erythrocytes, and in vitro mixtures was determined with carboxypyridine disulfide (CPDS) (Grassetti et al., 1975). A sample equivalent to 0.5 mL of whole blood or plasma was mixed under nitrogen with 1.0 mL of 0.24 mg/mL CPDS and adjusted to a volume of 10/.0 mL with isotonic, pH 7.2, 0.15 M phosphate buffer. A blank solution was prepared by diluting the same volume of sample to 10.0 mL with buffer. The mixture was stoppered under nitrogen and gently shaken for 5 min at room temperature. The samples were centriguged, and the absorbance of the supernatant phase was measured at 344 nm against the blank. The molar extinction coefficient was used to determine the sulfhydryl concentration of the samples.

The reaction rate of hymenoxon with whole blood was determined by mixing 1.0 mL of whole blood with 2.0 mL of isotonic, pH 7.4, 0.1 M phosphate buffer and equilibrating at 37 °C for 10 min. The reaction was initiated by adding 0.23 mg of hymenoxon to the buffered blood. The in vitro reactions were allowed to proceed at 37 °C for 15, 60, 90, 120, or 180 min. The reactions were terminated by extracting the free hymenoxon into 6.0 mL of ethyl acetate. Triplicate samples were incubated for each time period. The concentration of unreacted hymenoxon was quantified by gas chromatography (Hill et al., 1979), and the reaction rate was determined by plotting the reciprocal of the change in hymenoxon concentration vs. reaction time.

Hymenoxon reacts readily with cysteine. Whole blood was treated with various inhibitors of sulfhydryl groups and then reacted with hymenoxon to ascertain if hymenoxon would react with sulfhydryl groups in blood. A few milligrams of Hg_2Cl_2, 4.0 mL of isotonic 1.2 μmol/mL *p*-(hydroxymercuri)benzoate (pHMB), 4.0 mL of 2.6% $AgNO_3$, or 4.0 mL of isotonic saline (control) was added to individual tubes containing 4.0 mL of whole blood. Six milliliters of CPDS in isotonic saline (0.24 mg/mL) was added to 3.0 mL of whole blood. The solutions were mixed gently and allowed to react at room temperature for 15 min. Then 1.06 mg of hymenoxon in isotonic saline was added to each solution. The solutions were stoppered and shaken gently for 3.5 h. The sulfhydryl concentration was determined for all of the reaction mixtures with the exception of the $AgNO_3$ medium. The addition of $AgNO_3$ caused lysis of the erythrocytes with the precipitation of silver salts which interfered with the sulfhydryl assay. Aliquots of the reaction mixtures were assayed for unreacted hymenoxon by GLC.

The effect of pH on the reaction of hymenoxon with blood was studied by reacting hymenoxon with blood at different hydrogen ion concentrations. Three milliilters of 0.25 M phosphate buffer (pH 5.0, 6.0, 7.0, 8.0, or 9.0) was mixed in duplicate with 0.5 mL of whole blood. Hymenoxon (0.13 mg) was added to each mixture, and the solutions were allowed to stand for 1 h at ambient temperature. The pH of each mixture was measured, and then the samples were assayed for hymenoxon.

The reaction of hymenoxon with plasma and resuspended washed erythrocytes was measured in an effort to localize the reactive site of hymenoxon in whole blood. Plasma was separated from the cells by centrifuging whole blood at 2500*g* for 10 min. The plasma was removed from the cells and stored under nitrogen. The erythrocytes were washed 5 times with isotonic saline and then resuspended

in isotonic saline to the original whole blood volume. Plasma (3.0 mL) or resuspended washed erythrocytes (3.0 mL) were mixed with 3.0 mL of isotonic saline (control), pHMB (1.2 μmol/mL), or 2.6% $AgNO_3$. The solutions were allowed to stand at room temperature for 15 min. Hymenoxon in isotonic saline was added to each mixture, and then each solution was shaken for 1 h at ambient temperature. At the end of the incubation, each sample was analyzed for free sulfhydryl content. In addition, each reaction mixture was assayed in duplicate for hymenoxon.

Resuspended washed erythrocytes were mixed with isotonic saline or water to determine the effect of cell lysis on the reaction with hymenoxon. The erythrocytes that were mixed with water were shaken vigorously to lyse the cells. Each reaction mixture was prepared in triplicate and incubated at room temperature for 30 min. The reaction was terminated by extracting with 6.0 mL of ethyl acetate, and the ethyl acetate extract was analyzed for hymenoxon by GLC.

Hymenoxon in Blood and Urine of the Rabbit Given an Intravenous Administration of Hymenoxon. A female New Zealand rabbit was given 63.5 mg of hymenoxon in 1.5 mL of Me_2SO via the marginal ear vein. Blood samples were collected preadminstration and 7 min postadministration. The rabbit was killed 5.5 h after receiving the hymenoxon by disarticulating the cervical vertebrae, and the urine was collected from the urinary bladder. The clotted blood was centrifuged for 5 min after collection. Sera (0.5 mL) and urine (1.0 mL) samples were extracted with 6.0 mL of ethyl acetate. An aliquot of the ethyl acetate was evaporated to dryness, and the residue was dissolved in 50 μL of ethyl acetate. A 2.0-μL sample of the concentrated extracts was analyzed on the Varian GC at an attenuation of 64×10^{-12} A. A solvent blank was carried through the same procedure.

Distribution of [^{3}H]Hymenoxon in the Rabbit after Intravenous and Oral Administration. Purified [^{3}H]hymexnoxon was prepared for iv and oral administration by diluting 0.2 mL of the 1.04 mCi/ML solution of [^{3}H]hymenoxon with 2.0 mL of unlabeled hymenoxon (41.57 mg/mL) in Me_2SO (94.5 μCi/mL; 37.79 mg/mL unlabeled hymenoxon). A dose of 43.0 μCi/kg (17.2 mg/kg) of [^{3}H]hymenoxon was administered to two female New Zealand rabbits (3.3 and 3.2 kg) via the marginal ear vein. The same dose was given orally via a stomach tube to a female New Zealand rabbit (3.4 kg) that had been fasted for 48 h. The rabbits were placed in restrainers, and heparinized blood samples were collected from each rabbit at 0, 0.08, 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0, and 5.5 h post administration. The rabbits were held in the restrainer for 5.5 h and then killed by disarticulation of the cervical vertebrae. The rabbits were immediately necropsied. The total volume of urine in the urinary bladder and bile in the gall bladder was collected and measured. Duplicate tissue samples from the liver, kidney, lung, and brain and contents from the stomach and intestinal tract were collected immediately and weighed into combustocones.

Standard [^{3}H]hymenoxon solutions were prepared in duplicate for tritium analysis in the Tri-Carb sample oxidizer. The counts per minute per nanocurie of each standard was determined and used to determine the tritium activity of tissue samples.

Body fluid samples were assayed directly for tritium by adding an internal standard to correct for quenching effects. Aliquots (10 μL) of blood, urine or bile were mixed with 10 mL of Riafluor scintillation cocktail and 100 μL of Me_2SO, and then the solutions were assayed for tritium activity. An internal standard solution of [^{3}H]hymenoxon was added to each sample, and the scintillation mixture was recounted. The relative activity of the unknown sample to the internal standard was calculated by dividing the counts per minute of the unknown sample by the counts per minute of the internal standard per nanocurie of internal standard. By use of the same procedure, a standard curve for tritium activity was established by plotting the relative activity of [^{3}H]hymenoxon vs. nanocurie of standard. The slope of the line was used to calculate the tritium activity of the body fluid samples.

Metabolites of Hymenoxon. Urine and bile collected from a rabbit given [^{3}H]hymenoxon iv were analyzed by TLC to determine the metaboites of hymenoxon. Bile (50 μL) and urine (100n μL) were evaporated to dryness at 64 °C in a stream of nitrogen, and the residue was dissolved in 150 μL of methanol. For an assay of the presence of glucuronides, 100 μL of urine or 50 μL of bile was incubated with 500 μL of 3000 units/mL β-glucuronidase (Sigma) in pH 4.8, 0.1 M acetate buffer at 37 °C for 12 h and at room temperature for 24 h. The reaction mixtures were evaporated to dryness under a stream of nitrogen of 64 °C, and the residue was dissolved in 150 μL of methanol.

A 30-mL aliquot of each methanol extract of bile, urine, and β-glucuronidase-treated bile and urine was applied as a separate spot on a 0.5-mm thick silica gel G TLC plate. On another plate, 30 μL of methanol extracts of urine and bile and 50 μL of the hymenoxon standard were applied as separate spots. The TLC plates were developed to 15 cm in a solvent system of butanol–acetic acid (5:1.2) saturated with water. The position of ^{3}H-labeled compounds was determined by scraping consecutive 0.5-cm bands of the silica gel from the individual chromatograms into a scintillation vial and assaying each band for ^{3}H activity. Radiochromatograms were constructed by plotting the percent of the total ^{3}H activity in each band vs. the mean distance of the band from the origin.

RESULTS AND DISCUSSION

In Vitro Reaction of Hymenoxon with Blood. The extraction of hymenoxon from blood with ethyl acetate was poor, and the percent recovery decreased with an increase in incubation time. This suggested that the hymenoxon was reacting with some component of the blood. A reciprocal plot of the concentration of hymenoxon vs. reaction time (Figure 2) gave a good linear correlation coefficient ($r = 0.998$) for a line with a slope of 73.1 L/(mol min), which indicated that the reaction was second order (Barrow, 1961).

Hymenoxon reacts readily with the sulfhydryl group of cysteine. The sulfhydryl inhibitors (Hg_2Cl_2, pHMB, and CPDS) decreased the concentration of the extracellular sulfhydryl groups of blood by 90% but did not inhibit the reaction of hymenoxon with blood as the free hymenoxon content was less than 90% of the amount added to the whole blood. Silver nitrate decreased the reaction rate of hymenoxon with blood to 85% of the control reaction rate (Table I). The hydrogen ion concentration affected the reaction rate of hymenoxon with whole blood (pH 8 > 7 > 5). The enhanced reactivity at pH 8 is consistent with the reaction of sulfhydryl groups which are more ionized at a basic pH. The reaction can occur both enzymatically and nonenzymatically. This type of reaction is common to α, β-unsaturated carbonyl compounds reacting with glutathione (Clapp and Young, 1970; Chasseaud, 1973). However, the sulfhydryl inhibitors did not affect the reaction, which indicated that the principal reaction of hymenoxon in blood was not with sulfhydryl groups. Heat-denatured, whole blood reacted with hymenoxon at a level of only 60% of the nondenatured whole blood,

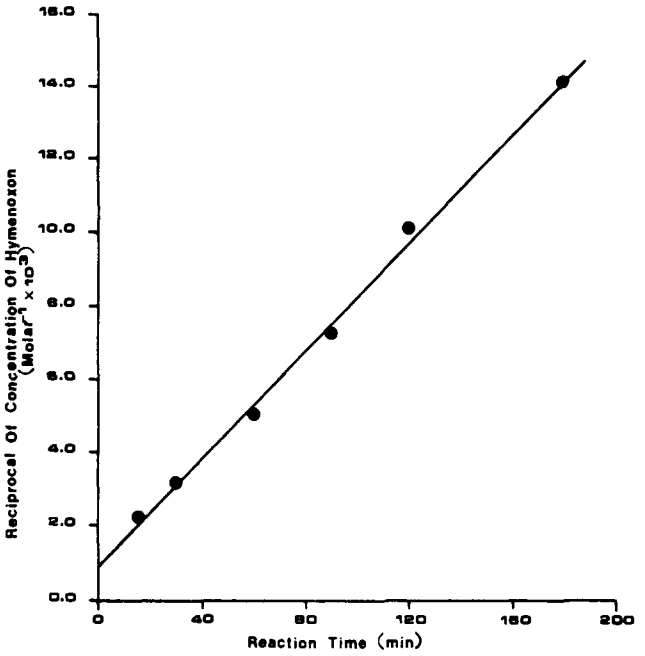

Figure 2. The in vitro reaction of hymenoxon with whole sheep blood at 37 °C. The rate is expressed as the reciprocal concentration of free hymenoxon vs. reaction time.

Table I. Recovery of Hymenoxon from Whole Sheep Blood Pretreated with Sulfhydryl Inhibitors

treatment	SH concn, μmol/mL	recovery,[c] %
whole blood[a]	0.338	
control[b]	0.358	<10.0
saline	0.358	<10.0
Hg_2Cl_2	0.048	<10.0
pHMB	0.060	<10.0
CPDS	0.507	11.0
$AgNO_3$		85.4

[a] Nontreated. [b] Hymenoxon treated only. [c] Recovery after 0.227 mg of hymenoxon stood in 1 mL of whole blood for 3.5 h.

suggested that the reaction was partially catalyzed by enzymatic activity.

The recovery of hymenoxon from sheep plasma and ertythrocytes after a 1-h reaction period was 95 and 51%, respectively. In a separate study, the recoveries of hymenoxon from sheep erythrocytes following a 30-min reaction period were as follows: erythrocytes, 70.9%; lysed erythrocytes, 75.8%; lysed erythrocytes without membrane fragments, 78.4%. From these results, it could appear that hymenoxon reacts with the erthyrocyte fraction of the blood and that the membrane is not the primary site of reaction. The importance of this reaction in relation to the toxicity of hymenoxon is not known. The erythrocyte may be responsible for the sequestering of hymenoxon in blood.

Detection of Hymenoxon in Blood and Urine of the Rabbit. Analysis of concentrated ethyl acetate (80×) at an attenuation of 64 × 10^{-12} A on the Varian GC showed the presence of an extraneous peak (solvent contaminant) at the point where hymenoxon eluted. In the presence of the solvent contaminant, the lowest level of hymenoxon that could be detected was 104 ng.

Free hymenoxon could not be detected in 0.5 mL of serum from the rabbit 7 min after the iv administration of 17.2 mg/kg hymenoxon. The urine of the rabbit contained 2.6 μg/mL free hymenoxon at 5.5 h postadministration.

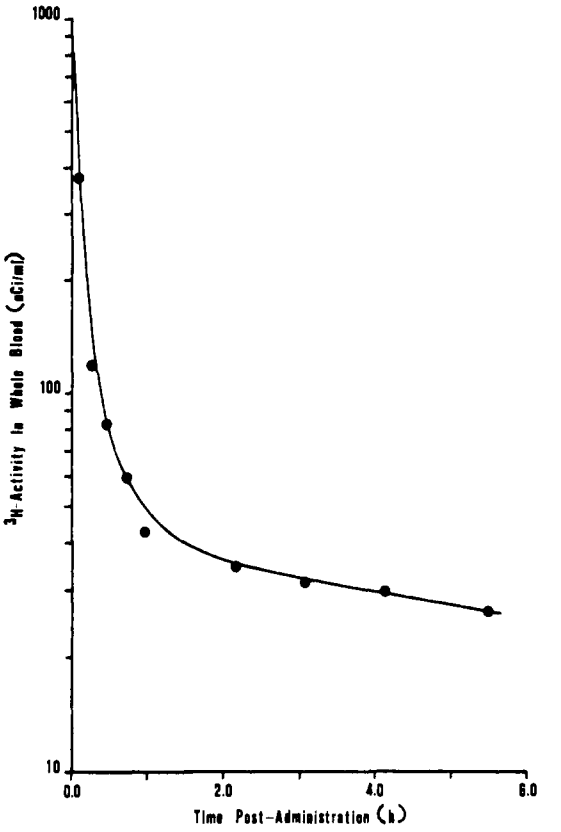

Figure 3. Biological decay of tritium from blood of rabbit 5 following a 43.0 μ Ci/kg iv administration of [^{3}H]hymenoxon.

Distribution of [^{3}H]Hymenoxon in the Rabbit. The logarithmic disappearance of tritium from the blood of two rabbits given an iv dose of [^{3}H]hymenoxon showed a triphasic relationship with time. The biological decay constant and the biological half-life for the three segments of the curve were identical for the two rabbits (Figure 3). The elimination of tritium from the blood 2 h after the iv administration was slow ($t_{1/2}$ = 9 h). The equation for data presented in Figure 3 for rabbit 5 was $C_t = 241.6e^{-10.75t} + 60.1e^{-2.70t} + 16.2e^{-0.077t}$, where C_t equals the tritium activity of blood at time t in nanocuries per milliliter. The equation for the decay of tritium in the blood of rabbit 6 was $C_t = 175.5e^{-10.83t} + 99.8e^{-2.87t} + 14.1e^{-0.0767t}$. In the rabbit given the oral dose of [^{3}H]hymenoxon, tritium was first detected in the blood 0.5 h posttreatment and the blood level of tritium was still increasing at 5.5 h posttreatment.

The body distribution of tritium vaied with the route of administration. At 5.5 h postadministration, the fraction of administered tritium present in the major organs of the rabbits given the labeled compound iv and per os was 4.7 and 0.4%, respectively. Only 50% of the administered tritium was absorbed from the GI tract when the compound was given orally. The ^{3}H activity of the blood was 4.7 and 0.6% of the administered tritium for the iv and oral treatments, respectively, whereas the activity in the brain was the same for each treatment. Of the tissues sampled, the greatest concentration of tritium was found in the lungs with the lowest activity associated with the brain.

Metabolites. Radiochromatograms of urine and bile revealed the presence of five and six compounds containing tritium in the bile and urine, respectively (Table II). There were four compounds in each fluid that possessed similar R_f values. Bile contained a compound (R_f 0.12) that was not observed in the radiochromatogram of the urine, while the urine contained two compounds with high R_f

Table II. R_f Value of Tritium-Labeled Compounds from Bile and Urine of a Rabbit Given [^{3}H]Hymenoxon Intravenously

	bile		urine	
conditions	R_f	relative act., %	R_f	relative act., %
nontreated	0.12	14.4	0.28	8.9
	0.25	16.8	0.35	23.3
	0.38	26.5	0.45	13.4
	0.45	23.2	0.60	30.0
	0.55	19.0	0.69	9.9
			0.79	14.4
treated[a]	0.15	42.2	0.15	32.0
	0.24	29.2	0.29	57.1
	0.35	13.1	0.50	6.9
	0.45	7.0	0.66	4.0
	0.51	5.5		
	0.61	3.0		
hymenoxon	0.82		0.82	

[a] Hydrolyzed with β-glucuronidase at pH 4.8 for 24 h.

values that were not detected in the bile. One of the compounds in the urine had an R_f value (0.79) comparable to that of free hymenoxon; however, the compound could not be detected after the urine was treated with β-glucuronidase.

When bile was treated with β-glucuronidase, the radioactivity of each spot on the chromatogram diminished with the concomitant appearance of a new, highly active spot (R_f 0.15). It was concluded that different glucuronides had a common aglycon. From the lower R_f value of the newly formed compound, the aglycon would appear to be more polar than the glucuronide.

The TLC profile of the urine changed when the urine was treated with β-glucuronidase. All of the high R_f value spots disappeared; however, the spot at R_f 0.27–0.30 broadened and increased in concentration. This evidence suggests that the metabolites in the urine were glucuronides, and from relative R_f values it would appear that similar derivatives appeared in the bile. There was evidence to suggest that two additional glucuronides were present in the urine and that the aglycon for these compounds was different from that of the glucuronides of the bile.

GLC analysis of urine from a rabbit given a 17.2 mg/kg iv dose of hymenoxon indicated that approximately 154 μg of free hymenoxon was excreted in 5.5 h. This represents only 1.6% of the estimated hymenoxon biotransformation products excreted in 5.5 h in the rabbits that received the iv dose of [^{3}H]hymenoxon. These data would suggest that hymenoxon was conjugated as glucuronides and then excreted via the bile and urine.

CONCLUSIONS

Reaction of Hymenoxon with Blood. Hymenoxon reacted with one or more endogenous compounds in blood. A primary reaction was associated with the erthyrocytes and was probably localized within the cell. Although hymenoxon reacts readily with cysteine, the evidence suggested that the reactive site in the blood was not sulfhydryl groups. The reaction hymenoxon with blood was partially inhibited by heat denaturation, which suggested that part of the reaction was catalyzed by enzymes. Sufficient evidence was not obtained to propose the type of reaction that occurs within the ertythrocyte.

Disposition of Hymenoxon in the Rabbit. Hymenoxon was slowly absorbed from the GI tract of the rabbit and was rapidly biotransformed and excreted in the bile and urine. The evidence indicated that all of the metabolites that were detected in the urine and bile were glucuronides of two different aglycons. Hymenoxon could have formed ether-type glucuronides; however, hymenoxon was not detected in the urine or bile after treatment with β-glucuronidase. The aglycons could have been oxidation products of hymenoxon in which one or both of the hydroxy functional groups were converted to carboxy functional groups. Ether-type (conjugation with hydroxy functional group), ester-type (conjugation with carboxy function group), or enol-type (conjugation with lactone function) glucuronides might be formed from these aglycons. Moreover, under basic conditions, hymenoxon can be converted to psilotropin. However, psilotropin was not detected in the urine or bile samples.

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Received for review December 10, 1979. Accepted June 13, 1980. This work was supported in part by the Texas Agricultural Experiment Station Project No. H-6255, Texas A&M University System. Technical Article No. 15653.