Identification and Toxicological Effects of Dehydroretronecine, A Metabolite of Monocrotaline[1] (37693)

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The pyrrolizidine alkaloids (PAs) produce quite diverse effects on the tissues of experimental animals (1, 2). However, these alkaloids are chemically inert and become toxic only after being metabolized (3). They are metabolized by hydrolysis of the ester group (4), by formation of *N*-oxide (5), and by dehydrogenation to pyrrole metabolites (3, 6). Indications are that the pyrrole metabolites are primarily responsible for the toxicological changes produced by the pyrrolizidine alkaloids (7, 8). Following *in vitro* incubation of the PAs lasiocarpine or heliotrine with rat liver microsomes, dehydroheliotridine (6,7-dihydro-7α-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine) has been isolated and identified as the major pyrrole metabolite (9).

In the present study, the identification and toxicological effects of dehydroretronecine (6,7-dihydro-7β-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine), the major pyrrole metabolite of monocrotaline, is presented. This metabolite is found in large quantities in the liver, blood, and urine of rats given monocrotaline. When dehydroretronecine is administered, it produces toxic effects in the liver of rats similar to those caused by the parent PA.

Materials and Methods. A number of separate experiments were required for identification and evaluation of dehydroretronecine. Dehydroretronecine was prepared from monocrotaline (S. B. Penick and Co., New York, NY) by barium hydroxide hydrolysis of monocrotaline to retronecine (10). Retronecine was then converted to dehydroretronecine by dehydrogenation with chloranil (11).

For the identification of dehydroretronecine in the urine, monocrotaline (100 mg in 1 ml aqueous solution, pH 7), was injected subcutaneously into four 450-g Sprague–Dawley rats which were housed in metabolic cages. Urine was collected during the subsequent 24 hr, saturated with K_2CO_3, and extracted with chloroform (40 ml $\times$ 3). The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness with a flash evaporator. The residue as well as a known sample of dehydroretronecine were separately dissolved in 1 ml of chloroform and applied on a silica gel plate. The plates were developed in a solvent containing $CHCl_3$:MeOH:NH_3 (85:14:1). Pyrrole derivatives were detected by spraying with Ehrlich's reagent (12).

In order to evaluate the presence of dehydroretronecine in liver and blood, 6 rats weighing 450 g received 75 mg/kg body weight phenobarbital subcutaneously daily for 4 days followed on the fifth day by 200 mg/kg body weight of monocrotaline. This pretreatment regime has been shown to enhance hepatic microsomal enzymes and thereby increase the metabolism of monocrotaline (13). The rats were decapitated 1.5 hr after the monocrotaline injection when pyrrole levels were at a maximum level in the blood and tissues (13). Five milliliters of blood and fourteen grams of liver were obtained from each animal and blended separately with 3 vol of acetone. A clear acetone extract was obtained after centrifugation and

[1] This investigation was supported in part by U.S. Public Health Service Grants HL-10941 and RR-00167 from the National Institutes of Health and The American Cancer Society Institutional Research Grant IN-35M-11. Primate center publication No. 13-019.

was evaporated at 40° to 3–5 ml. The latter was then saturated with potassium carbonate and extracted with chloroform (5 ml × 3). After removing moisture with anhydrous sodium sulfate, the combined chloroform extracts were evaporated to a dry residue and transferred to a test tube by washing with acetone. The volume of acetone was reduced to 0.3 ml by blowing with nitrogen gas. An aliquot of this solution and a known standard was subjected to thin layer chromatography.

The acute toxic effects of dehydroretronecine were evaluated on 8 rats weighing 90–100 g. The rats were injected subcutaneously with 110 mg/kg body weight of dehydroretronecine (60 mg/ml phosphate buffer, pH 7). Four control animals were given a similar injection of phosphate buffer. Fourteen hours after injection, a partial hepatectomy was performed (14). Thirty-two hours following surgery, the rats were given 1 mg/kg body weight of colchicine subcutaneously and killed 6 hr later. Portions of the regenerative liver were placed in Carnoy's fixative and subjected to an acetocarmine squash procedure for evaluation of mitotic index (15). At least 3,000 hepatocytes from each liver sample were observed in successive high-power microscopic fields in order to determine the number of colchicine-arrested metaphases present per 1,000 hepatic cells.

In order to establish the subacute effects, 14 rats weighing 70–90 g were injected subcutaneously with 70 or 90 mg/kg body weight of dehydroretronecine weekly for 6 successive weeks. Another 10 rats served as controls and received similar injections of phosphate buffer. After 12 weeks, the surviving rats were partially hepatectomized and treated in a manner similar to that described above for evaluation of mitotic index.

A complete necropsy was performed on all rats. Tissues from all organs including surgical and regenerative liver were placed in neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results. Dehydroretronecine was shown to be the major detectable pyrrole metabolite in the liver, blood, and urine of rats injected with the retronecine-based PA monocrotaline (Fig. 1). While no Ehrlich-positive pyrrole spot was present at the origin or at R_f values below 0.2, a relatively strong unidentified spot with an R_f value about 0.7 was observed in the urine samples.

The acutely intoxicated rats became listless and inactive shortly after receiving 110 mg/kg body weight of dehydroretronecine. The hepatic mitotic indices of the experimental group were approximately 1/30th the value of the control animals (Table I). At necropsy, the stomach was markedly distended with a large quantity of watery fluid in all of the treated animals. Microscopically, there was marked edema of the gastric submucosa. Reduced spermatogenesis and a marked decrease in the size and weight of the thymus and spleen were present in all of the treated rats. Microscopically, it was determined that the decreased size of the thymus and spleen resulted from reduced numbers of cortical thymocytes and regression of the splenic lymphoid nodules. Changes in the hepatic tissue obtained at the time of surgery were limited to a moderate infiltration of fat into parenchymal cells. Grossly and microscopically, other body organs were unaltered.

In the subacute experiment, the dehydroretronecine-treated rats experienced a slightly reduced rate of growth. Seven rats which had received 90 mg/kg body weight of dehydroretronecine died within 30 days of the initial injection. Multiple areas of focal ulceration and hemorrhage in the glandular portion of the stomach were present in these animals (Fig. 2). No other gross abnormalities were observed at necropsy in the animals that died or those sacrificed at 12 weeks. However, microscopic examination of the liver revealed widely dispersed megalohepatocytes and focal areas of subcapsular scarring. Regenerative hepatic mitotic indices were approximately 1/13th that of the control animals (Table I).

Discussion. In the past it was somewhat perplexing that a highly stable compound such as a PA could be so toxic. It has been clearly demonstrated that a number of substances such as dimethylnitrosamine (16) and methylated aminoazo dyes (17) which

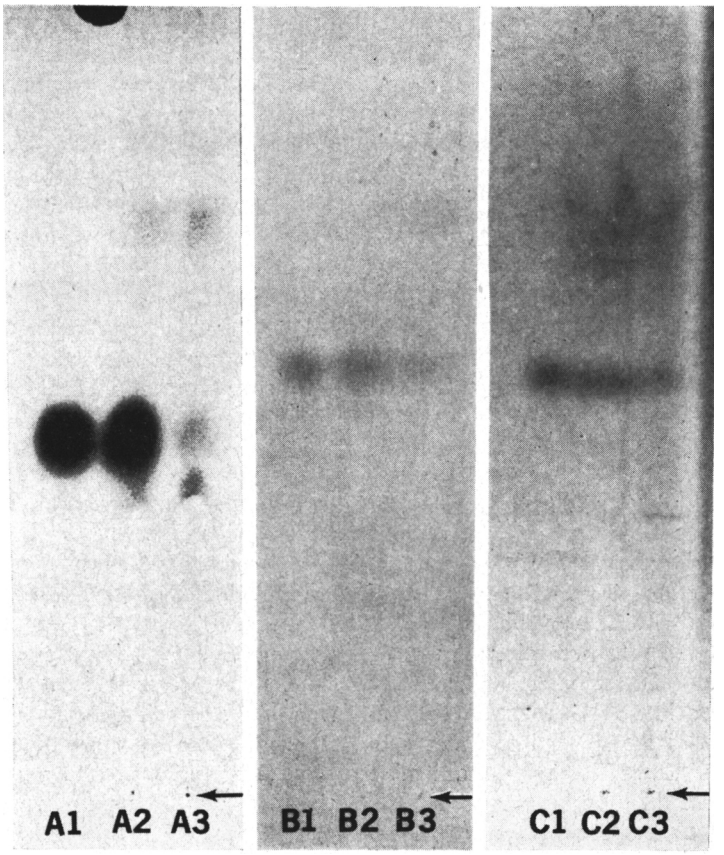

FIG. 1. Thin layer chromatography of urine (A), liver (B), and blood (C) samples from rats treated with monocrotaline. The samples were prepared as outlined in the text. A1, B1, and C1 are chromatograms of standard dehydroretronecine. A2, B2, and C2 are cochromatograms of dehydroretronecine and urine, liver, and blood samples. A3, B3, and C3 are chromatograms of urine, liver, and blood samples. Arrow indicates where samples were applied.

are harmless in themselves may be converted to toxic metabolites. Such a mechanism apparently accounts for the toxicity of the PAs. Many metabolites of these alkaloids have been identified (4, 12); however only the Ehrlich's reagent-positive metabolites, known as metabolic pyrroles, have been shown to be responsible for the toxicity (7, 8).

Chemically prepared monocrotaline and retronecine pyrrole inhibit mitosis and produce lung and liver lesions similar to those

TABLE I. Inhibition of Cell Division in Regenerating Liver of Dehydroretronecine-Injected Rats.

Experiment	Cumulative mitotic index	
	Control	Treated
ACUTE: sacrificed 52 hr after receiving 110 mg/kg	72.4 ± 16.9 (4)	2.5 ± 3.1[a] (4)[b]
SUBACUTE: sacrificed 12 weeks after receiving initial dose	67.6 ± 5.6 (6)	4.9 ± 2.5[a] (5)

[a] Analysis using Student's t test demonstrated that the difference between group means is statistically significant ($p < 0.001$).

[b] Number in parentheses indicates number of rats evaluated.

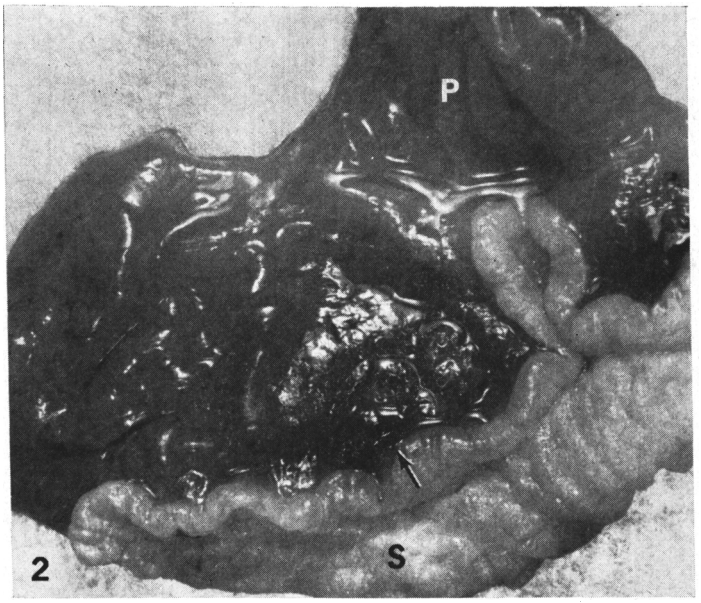

FIG. 2. Petechial hemorrhages (left) and ulceration (arrow) seen in the glandular portion of the stomach in a rat 8 days after receiving 90 mg per kg body weight of dehydroretronecine. S and P indicate squamous and pyloric portions of the stomach, respectively.

of the parent alkaloid (18, 19). These chemically prepared pyrroles are different in their optical absorption spectra from the *in vivo* pyrrolic metabolites and have not yet been demonstrated to exist in the body.

The precise means by which monocrotaline is converted *in vivo* to dehydroretronecine is not known. However, it seems likely that it is first dehydrogenated to form monocrotaline pyrrole and subsequently hydrolyzed to dehydroretronecine (9). Even though dehydroretronecine is not as toxic as monocrotaline pyrrole, it is the major detectable pyrrole metabolite and has been shown to be capable of producing lesions in the liver that are similar to those caused by the parent alkaloid.

Peterson and colleagues reported the toxicological effects of dehydroheliotrine (20). This metabolite is an enantiomer of dehydroretronecine and is capable of producing widespread toxic effects in experimental animals. However, the lesions produced differ considerably from those observed in dehydroretronecine-treated rats. While acute ulceration and hemorrhage of the gastric mucosa predominated in the animals given dehydroretronecine, similar tissue changes were inapparent in the dehydroheliotridine-treated rats. Alopecia, focal necrosis of the salivary glands, and abnormal development of the teeth were features only of dehydroheliotridine toxicity. Reduced hepatic mitosis, thymic regression, splenic hypoplasia, and inhibition of spermatogenesis were seen in animals treated with either of the PA metabolites.

The consistent production of gastric ulcers and hemorrhage in the stomach of rats given dehydroretronecine is an interesting previously unreported lesion. Preliminary studies have demonstrated that subcutaneously injected radioactive dehydroretronecine localizes in the stomach wall quite rapidly and persists at a level greater than other organs for an indefinite period. The most logical explanation for the localization of dehydroretronecine is related to the acid pH of the glandular portion of the stomach wall. Perhaps this compound is precipitated or activated in an acid environment, thereby concentrating the metabolite at this site. Once localizeed in sufficient concentration, it is probably capable of producing the tissue necrosis that invariably occurs in the stomach wall of dehydroretronecine-intoxicated rats.

Summary. The major detectable pyrrole

metabolite of the pyrrolizidine alkaloid, monocrotaline, present in the liver, blood, and urine of rats, has been identified as dehydroretronecine. The subcutaneous injection of this metabolite into rats produces ulceration and hemorrhage of the stomach wall. Inhibition of hepatic parenchymal cell mitosis in regenerating liver was recorded within 52 hr following its administration and persisted for at least 6 weeks.

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Received July 23, 1973. P.S.E.B.M., 1973, Vol. 144.