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Metabolic Conversion of Heliotridine-Based Pyrrolizidine Alkaloids to Dehydroheliotridine

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SUMMARY

The heliotridine-based pyrrolizidine alkaloids lasiocarpine and heliotrine were metabolized *in vitro* by rat liver microsomes to one main chloroform-extractable and water-soluble pyrrolic derivative, which was isolated and identified as dehydroheliotridine (6,7-dihydro-7α-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine) by chromatography and nuclear magnetic resonance and mass spectroscopy. Only trace amounts of this metabolite were formed from the *N*-oxides of lasiocarpine or heliotrine, and it was not formed from supinine.

INTRODUCTION

The hepatotoxicity of pyrrolizidine alkaloids has been ascribed to the action of an "active" metabolite which is produced in the liver and which sometimes escapes into the circulating blood, causing lesions in other organs, such as the lung and kidneys (1–4). Products of metabolic hydrolysis, hydroxylation, and *N*-oxidation of these alkaloids were isolated by Dann (in ref. 1), but these metabolites retained a 1,2-dehydropyrrolizidine ring and were less toxic than the parent alkaloids (1). More recently, it has been shown that pyrrolic derivatives are produced *in vivo* and *in vitro* (3, 4). In the present study it was found that one main water-soluble and chloroform-extractable pyrrolic metabolite was produced from the heliotridine-based alkaloids lasiocarpine and heliotrine by rat liver microsomes *in vitro*. This metabolite was isolated and identified as dehydroheliotridine (6,7-dihydro-7α-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine).

MATERIALS AND METHODS

Enzyme preparations. Homogenates (20% in 0.154 M KCl) were prepared from the livers of adult male rats and centrifuged for 10 min at 15,000 × *g*, and the supernatant fractions (combined microsomal and soluble fractions) were used as "microsomal preparations." Such microsomal preparations were made both from the livers of untreated rats and from those of rats treated with three daily doses of 80 mg of sodium phenobarbital per kilogram of body weight, administered by intraperitoneal injection.

Reaction mixtures. Incubation mixtures contained 0.02 M KH_2PO_4 adjusted to pH 7.5 with 1 N NaOH, 5 mM $MgSO_4$, 10 μM $MnCl_2$, 10 mM glucose 6-phosphate, 0.1 mM NADP, 5 or 10 mM alkaloid, previously neutralized with dilute HCl, and micro-

somal preparation equivalent to one-half the total final volume, which was usually 0.5 ml. The mixtures were incubated at 37° with shaking. Incubation was conducted for 10 min for chemical assay (3, 5) and for 60 min for analysis by thin-layer chromatography. The plates (silica gel G) were developed in chloroform-methanol-ammonia (0.880 sp. gr.; 85:14:1 by volume) (solvent 1). Both samples of the reaction mixtures and chloroform extracts of these mixtures were subjected to thin-layer chromatographic analysis. The chromatograms were sprayed with Ehrlich's reagent.

Large-scale preparation. To obtain sufficient pyrrolic metabolite for identification, mixtures of 5 ml each were prepared using lasiocarpine as substrate, and a microsomal preparation was made from the liver of a rat treated with sodium phenobarbital. After incubation at 37° for 30 min, the mixtures were extracted with chloroform, and the pooled extracts were reduced in volume and applied to a preparative thin-layer chromatography plate. After development of the plate and location of the metabolite by a marker channel, the band of silica gel containing the metabolite was scraped off and eluted with methanol. The resulting methanol solution was evaporated to dryness, and the metabolite was taken up in methylene chloride. For measurement of the mass spectrum, a drop of this solution was evaporated in a tube for direct entry into the mass spectrometer (Hitachi Perkin-Elmer, RMU 6D). For measurement of the nuclear magnetic resonance spectrum, the remaining solution was evaporated and taken up in deuterochloroform. The spectrum was measured with a Varian HA-100 spectrometer and a Varian C-1024 computer and recorded as the average of 222 scans.

Detection of quaternary metabolites. Incubation mixtures of 5 ml each, similar to those of the large-scale preparation, were prepared using 10 mM lasiocarpine, heliotrine, or supinine as substrate. After incubation for 1 hr at 37°, each mixture was extracted with chloroform, and the extracts were dried over Na_2SO_4 and evaporated to small volumes (*X* extracts). The aqueous residues were saturated with $NaHCO_3$ and KCl and extracted further, using chloroform containing 5% methanol. The extracts were dried over Na_2SO_4 and evaporated to small volumes (*Y* extracts). Portions of the *X* and *Y* extracts were analyzed by both thin-layer and paper chromatography. The thin-layer chromatograms were developed as described above, and the paper chromatograms were developed in 1-butanol–acetic acid–water (80:3:17 by volume) (solvent 2). All chromatograms were sprayed with Ehrlich's reagent.

RESULTS

The three alkaloids lasiocarpine, heliotrine, and supinine, their respective *N*-oxides, and the amino alcohol heliotridine were incubated with rat liver microsomes, and the products of the reactions were analyzed by the chemical assay for the production of pyrrolic derivatives. Pyrrolic compounds were formed readily from the alkaloids, but not from the *N*-oxides or heliotridine. Values found, expressed as micromoles of pyrrole produced per gram of liver, wet weight, in 30 min, were 5.2 from lasiocarpine, 1.2 from heliotrine, and 1.4 from supinine. The use of microsomes from rats previously treated with sodium phenobarbital increased the rate of production of pyrroles from the alkaloid esters 4–5-fold. However, there was still no detectable transformation of the *N*-oxides or heliotridine.

Thin-layer chromatographic analysis of reaction mixtures containing lasiocarpine showed the presence of three Ehrlich-positive spots, with R_F values of 0.45 (A), 0.07 (B), and 0.0 (C). The major spot (A) had an R_F identical with that of an authentic sample of dehydroheliotridine (compound I), and both gave a purple color with the Ehrlich reagent. Co-chromatography of spot A with authentic dehydroheliotridine gave a single, discrete spot. Mixtures containing heliotrine as substrate yielded pyrrolic derivatives that had R_F values and colors similar to those of lasiocarpine, but the amounts of each were considerably smaller. Several pink-mauve spots were obtained from chloroform extracts of mixtures incubated with supinine, but their R_F values were different from those obtained from

lasiocarpine and heliotrine. Trace amounts of purple, Ehrlich-positive material (R_F 0.45) were obtained from chloroform extracts of mixtures incubated with lasiocarpine *N*-oxide, heliotrine *N*-oxide, and heliotridine. This indicated only a very small amount of conversion to pyrrolic substances, as thin-layer chromatography was about 5 times more sensitive than the chemical assay for the detection of dehydroheliotridine.

A sample of metabolite A was isolated by preparative thin-layer chromatography from larger reaction mixtures, in which lasiocarpine was incubated with a microsomal preparation from a rat that had been treated with sodium phenobarbital. The identity of this metabolite with dehydroheliotridine (compound I) was established by means of mass and nuclear magnetic resonance specta. The major peaks in the mass spectrum of authentic dehydroheliotridine are as follows: *m/e* 153, 136, 135, 134, 122, 118, 117, 107, 104, 94, 90. The mass spectrum of the metabolite as recovered from the thin-layer chromatography plate contained all these peaks at similar relative intensities, except that *m/e* 117 was much more intense than in the best reference spectrum. Other spectra of dehydroheliotridine also contained an intense peak at *m/e* 117, the relative peak intensities apparently varying with the conditions of measurement and the purity of the sample. A continuous background of low-intensity peaks was shown to be due to substances extracted from the silica gel of the thin-layer chromatography plate. Subtraction of a spectrum due to these substances, obtained in a blank run, from that of the extract containing the metabolite gave a residual spectrum which contained no peaks with more than 10% intensity that were not present in the reference spectrum of dehydroheliotridine. The nuclear magnetic resonance spectrum of authentic dehydroheliotridine in deuterochloroform at concentrations below 2% consists of broad multiplets in the $\delta = 2.5$ and $\delta = 4.0$ regions, which are due to the H-6 and H-5 protons, respectively; two strong lines near $\delta = 4.5$, which are the inner lines of an AB system due to the slightly nonequivalent H-9 protons; a four-line multiplet, $\delta = 5.13$, due to H-7; and two doublets, $\delta = 6.12$ and 6.48 ($J = 2.5$ cps), which are due to the pyrrolic protons, H-2 and H-3. At higher concentrations, the H-9 protons are of equal chemical shift, $\delta = 4.6$. The spectrum of the metabolite showed the doublets $\delta = 6.12$ and 6.48, the multiplet $\delta = 5.13$, the strong lines near $\delta = 4.5$, and the broad multiplets near $\delta = 2.5$ and 4.0.

The optical purity of the metabolite was not established. However, since the configuration at C-7 is apparently retained during the chemical oxidation of heliotridine to dehydroheliotridine (6), it is reasonable to assume that it is also retained during the metabolic formation of compound I from heliotridine-based alkaloids.

Another scaled-up reaction mixture containing heliotrine as substrate was extracted with chloroform, and the extracts were analyzed by thin-layer and paper chromatography to obtain evidence for the identification of a possible component of metabolite spots B and C. Paper chromatography of the *Y* extracts showed the presence of Ehrlich-positive spots of R_F 0.21 (mauve) and 0.50 (spot D; mauve, becoming dark gray on standing), as well as a small amount of dehydroheliotridine (confirmed by thin-layer chromatographic analysis) that polymerized on the origin. The R_F of spot D, its color, and change in color were identical with those given by *N*-[6,7-dihydro-7α-hydroxy-1-(5*H*-pyrrolizino)]methylheliotrine chloride (compound II) (7). Metabolite D and authentic compound II also showed identical R_F values and colors on thin-layer chromatograms developed in methanol (R_F 0.11) and in solvent 1 (R_F 0.0), although in the latter instance the spot could be displaced to form a crescent above other substances bound more firmly at the origin. Of the many pyrrolic and dihydropyrrolizine derivatives we have prepared chemically from the alkaloids, none except compound II and related quaternary substances derived from other alkaloids has an R_F value within the range 0.3–0.6 when chromatographed on paper using solvent 2.

Extraction and analysis performed in a similar manner on incubation mixtures containing lasiocarpine and supinine as sub-

strates gave evidence suggesting the formation of quaternary derivatives from these alkaloids, but such metabolites have not yet been completely characterized.

DISCUSSION

The main extractable pyrrolic metabolite produced by rat liver microsomes from the heliotridine-based pyrrolizidine alkaloids lasiocarpine and heliotrine was identified as dehydroheliotridine (I). This metabolite was not produced from supinine, an ester of supinidine (1, 8). The pyrrolic substances derived from supinine differed from the products of the heliotridine-based alkaloids in R_F values, in the colors of the Ehrlich-positive spots on chromatograms, and in the rate of color development in the chemical assay (9).

No pyrrolic derivatives were detected by chemical assay of incubation mixtures containing the alkaloid N-oxides, even when the microsomal preparations used were those from livers of rats treated with sodium phenobarbital, which is known to induce high levels of activity of the microsomal mixed-function oxidases (10) and markedly increased the rate of production of pyrrolic compounds from lasiocarpine and heliotrine. This strongly suggests that the N-oxides are not intermediates in the reaction(s) by which the alkaloids are converted metabolically into pyrrolic derivatives. Mattocks (3) found that "metabolic pyrroles" were formed from retrorsine N-oxide when incubated for 2–5 hr with rat liver slices. However, Dann (in ref. 1) showed that following intraperitoneal administration of heliotrine N-oxide to rats, 2.7% of the dose was excreted as heliotrine, and a further 2.7% as heliotridine trachelanthate. Thus, in the whole rat, at least, N-oxide is reduced back to the tertiary base, and if this reduction occurs in the liver, the reduced alkaloid so formed could be the origin of the pyrrolic compounds formed during incubation of alkaloid N-oxide with liver slices. The trace amounts of dehydroheliotridine shown by chromatography to have been formed from lasiocarpine N-oxide and heliotrine N-oxide

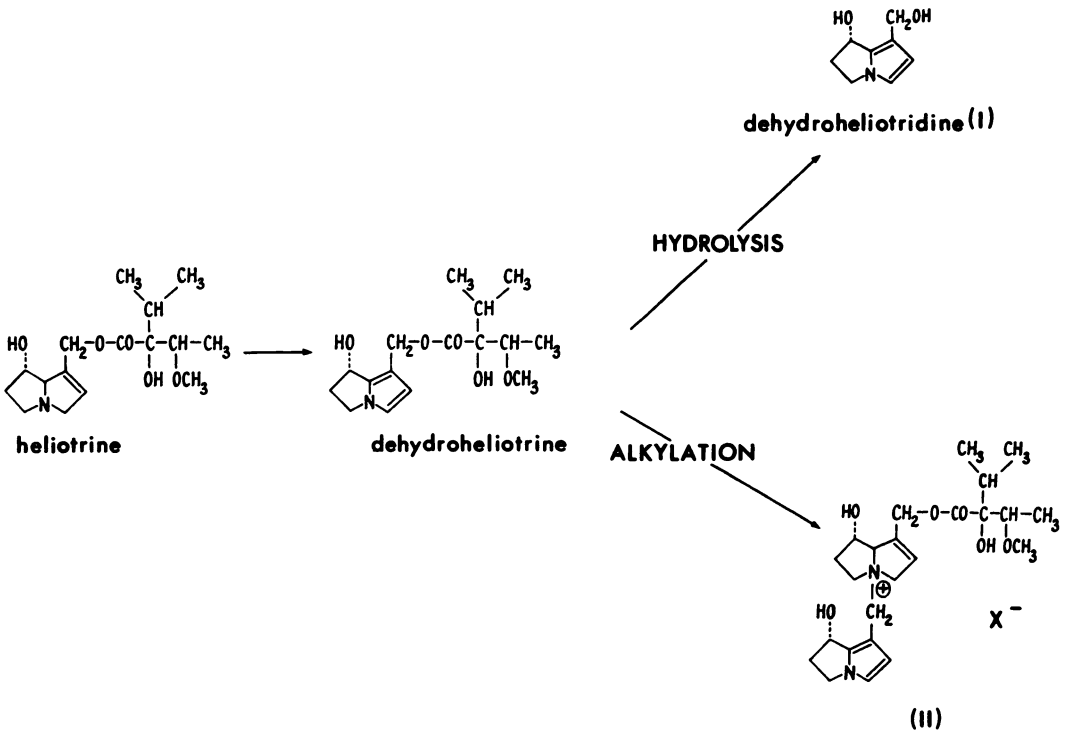

FIG. 1. *Possible mechanism for the metabolism of heliotrine to dehydroheliotridine*

by the microsomal preparations may also have arisen in this way.

A possible mechanism for the formation of dehydroheliotridine from heliotrine and lasiocarpine would be dehydrogenation of the heliotridine formed by hydrolysis of these alkaloids (1). However, as the rate of formation of dehydroheliotridine from heliotridine was very much lower than that from either lasiocarpine or heliotrine, it seems unlikely that the amino alcohol is an intermediate in the conversion of heliotridine-based pyrrolizidine alkaloids into dehydroheliotridine.

A more likely pathway for its metabolic formation is by hydrolysis of dehydroalkaloids. Evidence for the formation of dehydroalkaloids was provided by the identification of metabolite D from heliotrine as a salt of the quaternary compound II. This compound, which is relatively stable, is formed readily *in vitro* by interaction between dehydroheliotrine and heliotrine (12). Dehydroheliotrine and dehydrolasiocarpine, on the other hand, are extremely labile in aqueous solution (11), and hydrolyze spontaneously to give dehydroheliotridine and other, as yet unidentified products.[1] They are also highly reactive alkylating agents (4, 11). We have found that they combine readily with tertiary amines, including the parent alkaloid, and that quaternary compounds such as II are formed as an alkaloid undergoes chemical conversion to the dehydroalkaloid (7, 12). Although dehydroheliotridine is also an alkylating agent (4, 12) and thus is potentially able to act as a precursor for the metabolically produced quaternary compound II, it does not react with heliotrine at a sufficient rate to be a precursor of II under the conditions used in the present work.[1]

It therefore seems likely that, in the metabolism of heliotrine, the alkaloid is first converted by the microsomal enzymes of liver to dehydroheliotrine. The dehydroalkaloid then either alkylates suitable acceptors, such as unchanged heliotrine, proteins, and possibly nucleic acids, that are sufficiently close to the site of its formation, or rapidly undergoes hydrolysis to dehydroheliotridine (Fig. 1). Thus, the main extractable pyrrolic metabolite recovered was the relatively stable dehydroheliotridine. The results suggest a similar sequence of reactions during the metabolism of lasiocarpine and supinine. Dehydroheliotridine is also a major component of the pyrrolic derivatives excreted in the urine following administration of lasiocarpine or heliotrine (4). Apart from compound I, the other major Ehrlich-positive spot on thin-layer chromatograms of the unextracted reaction mixtures occurred at the origin. Probably a large proportion of this consisted of pyrrolic derivatives bound to microsomal or soluble proteins (3).

These metabolic processes apparently occur in plant cells also, as the quaternary compound II has been detected in extracts of *Heliotropium europaeum* (7).

[1] Unpublished observations.

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