METABOLISM OF PYRAZOLE
Structure Elucidation of Urinary Metabolites

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ABSTRACT

Pyrazole has been widely used as an inhibitor of alcohol dehydrogenase both in vitro and in vivo. Very little attention has been paid to the metabolism of this agent and possible biological activity of any metabolites. Several isotopic variants of pyrazole, both stable and radioactive, were used in a study of its metabolic fate by gas chromatography-mass spectrometry. Seven metabolites were structurally identified and included hydroxylated and conjugated derivatives of pyrazole. Two metabolites were conjugated with a pentose, perhaps indicating that pyrazole serves as a substrate in the salvage pathway of purines and pyrimidines forming pyrazole ribosides. The use of d3-pyrazole greatly enhanced structural assignment of the metabolites by revealing the metabolism at or next to a labeled carbon atom.

Pyrazole has been well characterized as a potent competitive inhibitor of hepatic alcohol dehydrogenase activity both in vitro (1) and in vivo (2). Recent reports suggest that metabolism of pyrazole produces compounds with biological activities quite different from ADH inhibition. Pyrazole does not inhibit catalase acutely in vivo or in vitro, but catalase is irreversibly inhibited approximately 24 hr after pyrazole administration to rats (3). Similarly, the activity of tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase) is not inhibited by pyrazole in vitro or at short times in vivo, but is inhibited by approximately 50% 8 hr after administration of pyrazole to rats (4). A pyrazole metabolite appears to be responsible for both of these effects.

Pyrazole metabolites appear also to be implicated in its profound toxicity. An acute lethal dose in rats (LD50) is approximately 15 mmol/kg (5), but doses which are not immediately lethal lead, after a period of a few days, to profound changes including liver necrosis, anemia, leukopenia, decreased spleen size, and death (5, 6). Pyrazole was evaluated in Phase I studies as an antitumor agent in man, but even in doses of 0.15 mmol/kg/day it proved too toxic for human use because of development of signs of hepatotoxicity after several days (7, 8).

Although its metabolism may provide an explanation for its toxicity, pyrazole metabolites have been studied very little. Pyrazole has been reported to be released in the urine of rats after treatment with glucuronidase/sulfatase (9), suggesting that glucuronide formation is a possible metabolic fate. With the exception of this report, however, the biotransformation of pyrazole has remained largely unexplored. Rydberg et al. (10) measured the plasma concentration of pyrazole after a single dose to rats, and found that elimination was a first-order process. In these studies, pyrazole had a half-life of 13 hr in plasma, which was increased to 21 hr after concomitant administration of ethanol. The probable implication of pyrazole metabolites as being biologically active molecules stimulated this study of the structural elucidation of pyrazole metabolites.
Materials and Methods

Synthesis of 3,4,5-Trideuteropyrazole. Pyrazole (1 g) was dissolved in 2.0 ml of D₂O (99.8 atom % BioRad Labs., Richmond, Cal.) containing 90 mg of NaOD. The solution was sealed in a glass tube and heated at 200°C for 24 hr. After cooling, the exchange reaction was stopped with 2 ml of H₂O and pyrazole was isolated by saturating the aqueous system with Na₂CO₃. A yellow oil appearing at the upper phase was extracted with diethyl ether and dried over Na₂SO₄. d₃-pyrazole was recrystallized from CC1₄. The yield was 62-64°C. 

Fresh d₃-pyrazole used in the metabolism studies was made by a modification of the above procedure. The initial exchange was made with 1 g of pyrazole, 10 ml of D₂O, and 90 mg of NaOD (BioRad) and heating at 100°C for 24 hr. The exchanged pyrazole was isolated by extraction without adding H₂O to stop the exchange. This product was then exchanged with fresh D₂O and NaOD. Mass-spectral analysis indicated 85 atom % d₃, 9 atom % d₄, 6 atom % d₅, with no d₆-pyrazole.

Synthesis of 3,4,5-Tritritopyrazole. Recrystallized pyrazole (0.5 g) was added to 0.9 ml of water containing 3.5 mg of KOH and 0.1 ml of H₂O (100 mCi/ml). The aqueous solution was sealed in a Pyrex tube and heated at 150°C for 48 hr. After cooling, 1 ml of H₂O was added, and the solution saturated with Na₂CO₃ and extracted three times with 5-ml portions of diethyl ether. The ether extracts were combined, back-extracted five times with saturated Na₂CO₃ solution, and the ether solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in 8 ml of H₂O, and Na₂CO₃ (1 g) and saturating amounts of NaCl were added. The solution was extracted three times with 10-ml portions of ether. The water layer was found to contain less than 1% of the radioactivity of the ether solution. Recrystallization of the product yielded 220 mg of very pure d₅-pyrazole with a specific activity of 4.87 µCi/mg (total 1.07 mCi).

Synthesis of [3,4,5-¹⁴C]Pyrazole. ¹⁴C-Acetylene gas (1.3 mg, 1 mCi) was dissolved in 19 ml of cold diethyl ether, yielding a total activity of 300 µCi. Diazomethane was added to the solution, which was sealed and placed in darkness for 5 days (11, 12). Fresh diazomethane was added at intervals as necessary. Unlabeled pyrazole (100 mg) was added to the ether solution, after which the ether was evaporated. The residue was recrystallized from petroleum ether. The first crop of crystals yielded 66 mg of very pure [3,4,5-¹⁴C] pyrazole, m.p. 63°C. The specific activity of the material was 2.39 x 10⁹ dpm/mg, a 26% overall yield from the transferred ¹⁴C-acetylene.

Synthesis of 1-Ribofuranosylpyrazole. Pyrazole (500 mg) was added to an ether solution of 2,3,5-tri-O-benzoyl-β-ribofuranosyl bromide which was made from 1-acetyl-2,3,5-tri-benzoyl-β-d-ribofuranoside (Calbiochem. San Diego, Cal.) (13). The solution was refluxed to eliminate HBr. The product was not isolated; however, after removal of the ether, 10 ml of methanol saturated with ammonia was added and refluxed for 4 hr. The product was analyzed by GC/MS after derivatization (BSTFA, CH₃CN, 60°C, 1 hr) and yielded a single chromatographic component aside from benzamide.

3-Hydroxypropyrazole. This compound (5-pyrazolone) was synthesized by the hydrazinolysis of uridine (14).

Metabolism Studies. Radioactive pyrazole (1 µCi per rat) was administered by ip injection of a saline solution into 10 mice (BALB/c) and 7 rats (Sprague-Dawley) in doses of 2-5 mmol/kg. Animals were placed in separate metabolic collection chambers; food and water were available ad lib. Urine and feces were collected at time intervals, at room temperature without preservatives, and total radioactivity excreted was determined by scintillation counting of aliquots of urine and feces. Feces were dissolved in NCS (Amersham/Searle Corp., Arlington Heights, Ill.). The internal standard method ([¹⁴C-toluene) was used to calculate the counting efficiency for each aliquot analyzed.

For one experiment, a mixture of the isotopic variants of pyrazole was made to a final concentration of 50% d₅-pyrazole and 1 µCi of [¹⁴C]-pyrazole. This was injected into a rat (ip) and the urine collected for 60 hr. The pooled urine (45 ml) contained 42% of the injected dose of pyrazole, based on the [¹⁴C]-content.

One-half of the urine from the d₅-pyrazole experiment was treated with 100 mg of urease (jack bean, Sigma Chemical Co., St. Louis, Mo.) at room temperature. Acetic acid was added to maintain pH 6, until gas evolution ceased. No radioactivity was lost from the urine during this treatment. The solution was evaporated to dryness and the residue treated with 50 ml of methanol. Silicar CC-7 (1 g, Mallinckrodt Chemical Works, St. Louis, Mo.) was added to the methanol solution and the methanol stripped off. The dried silica gel was added to the top of a packed silica gel column (20 x 1 cm) containing chloroform. The column was then sequentially washed with chloroform, diethyl ether, ethyl acetate, and a linear gradient of ethyl acetate to ethanol, with 40 ml fractions taken. The radioactivity in each eluate was determined and is listed in table 1.

Structure Determination of Pyrazole Metabolites. The fractions containing radioactivity from the column chromatography of the d₅-pyrazole experiment were analyzed by GC/MS. Aliquots of each sample were evaporated to dryness in a screw-capped culture tube, and 50 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, Bellefonte, Pa.) and an equal amount of acetoniitrile were added. After capping, the samples were heated to 60°C for 30 min. A Finnigan (Sunnyvale, Cal.) model 9500 gas chromatograph was used for the combined GC/MS. A 1.8-m glass column (2 mm I.D.) packed with 3% OV-17 liquid phase on Gas-Chrom Q (Applied Science Labs., State College, Pa.) was temperature-programmed from 70° to 300°C at 8°/min.; helium served as the carrier gas. Mass spec-
In order to evaluate the suitability of 3H-pyrazole for metabolism studies, three BALB-C mice were each administered 1.5 x 10^6 dpm of 2b and the urine collected and pooled at 3-day intervals. Only 40% of the injected tritium was recovered in the urine and feces. This observation appeared inconsistent with estimates of the t1/2 of pyrazole in this animal based on the duration of inhibition of alcohol dehydrogenase \textit{in vivo} (18). It was suspected that tritium was back-exchanging with the protons of water, or was being removed from the pyrazole nucleus by metabolism. This experience suggested that 14C-labeled pyrazole would be a more appropriate marker for pyrazole metabolism. Following the method of von Peckman (12) and Hückel et al. (11), 14C-pyrazole was easily synthesized by 1,3-dipolar addition of diazomethane to 14C-acetylene. Due to the resonance of pyrazole, the C3 and C5 positions become equivalent; thus every carbon position is labeled with 14C.

**Results**

The description of the base-catalyzed exchange of the protons of pyrazole (1) at positions C35 and C4 (15-17) stimulated us to consider complete exchange of all protons of this aromatic heterocycle by more extreme reaction conditions. 3,4,5-Trideuteropyrazole (2a) was successfully synthesized by employing basic conditions and heating at 200°C for 24 hr. This procedure was then used as a simple method to produce 3H-pyrazole (2b) with each carbon atom containing a tritium atom.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Per Cent of Total Radioactivity</th>
<th>Metabolites*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl3 (200 ml)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ether (200 ml)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate (200 ml)</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Ethanol gradient*</td>
<td>1, 2</td>
<td>4.4 A, J</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.2 A, J</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24.9 A, B, K</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21.6 A, B, C, K</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5.6 C, G</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>14.4 G</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<tr>
<td></td>
<td>11</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Codes for the structures discussed in the text.

40-ml fractions.
Percentage of the injected dose of $^{14}$C-pyrazole that had appeared in the urine of BALB/c mice (○) and Sprague-Dawley rats (●) following ip injection. The values are the means for 10 mice or 7 rats; brackets indicate ± SD.

**Fig. 2.** Reconstructed gas chromatogram.

Computer reconstruction (total ionization current) of the GC/MS analysis of derivatized fraction 5 (table 1). The abscissa indicates the mass spectrum index number to a total of 230 scans during the gas chromatogram. The arrows indicate the components that contained mass-spectral isotope doublets by computer analysis.

such a procedure would result in pyrazole metabolites displaying isotope doublets in their mass spectra and thus would be more easily detected in a complex mixture. Furthermore, the pooled urine was treated with urease to destroy urea prior to column chromatography. Although the column chromatography (table 1) did not cleanly separate each metabolite, there was sufficient separation to allow gas-chromatographic resolution and mass spectra to be recorded for seven separate metabolites of pyrazole. The GC/MS analysis of fraction 5 is shown in fig. 2 as an example of the complex mixtures. Also indicated in fig. 2 by arrows are those components in which computer analysis of the mass-spectral data revealed the presence of isotope doublets and which therefore are pyrazole metabolite candidates. This computer program for isotope-doublet detection greatly facilitated the analysis of the large amount of data generated in such a GC/MS study. For example, fig. 2 is the total ionization record of 230 separate mass spectra stored in the computer system.

The mass spectra of the TMS derivatives of two metabolites are presented in fig. 3. Both have an apparent molecular ion at $m/e$ 228, substantiated by the loss of a methyl radical (M−15) at $m/e$ 213. Each compound was silylated, as evidenced by the strong ion at $m/e$ 73 [(CH$_3$)$_3$Si$^+$]. Inasmuch as the TMS derivative of pyrazole would have a molecular weight of 140, each molecule is 88 mass units larger, which would correspond to the addition of oxygen and one TMS (minus hydrogen) to the pyrazole structure. The loss of one deuterium atom from d$_5$-pyrazole, as seen in the molecular ion of the doublet (inset) in fig. 3B, indicates hydroxylation at only one carbon atom of the pyrazole. A priori, it would be difficult to indicate the exact carbon hydroxylated; however, by comparing this mass spectrum with that of another hydroxylated metabolite shown in fig. 3A, the structure of this metabolite was assigned as 4-hydroxypyrazole (A). The mass spectrum of B showed a molecular ion at $m/e$ 228; however, there appeared to be a loss of two deuterium atoms, because the molecular ion doublet differed by only one mass unit. Explanation of the loss of
two deuterium atoms, although only one carbon is attacked, came from considerations of the chemistry of 3-hydroxy pyrazole. This molecule could undergo facile exchange of the deuterium atom at C4 because it is in equilibrium with the stable tautomer 5-pyrazolone. Substantiation of this structure B came from the strong ion at m/e 147, which is characteristic of compounds with two TMS groups in close proximity (20), which would be the case in 3-hydroxy pyrazole. 3-Hydroxy pyrazole was synthesized (13) and the mass spectrum of the TMS derivative was identical to that of metabolite B.

The remaining hydroxylated metabolite had an apparent molecular ion at m/e 332 (333) which is 16 mass units higher than that of the dihydroxy pyrazole derivative. The isotopic abundances (M+1, M+2, and M+3) were identical to those of metabolite C. These facts require the pyrazole metabolite to include three oxygen atoms, three derivatizable moieties, and one hydrogen atom left on a pyrazole-carbon atom. This eliminated structure F for example, which would result in the addition of four TMS residues and loss of all the deuterium label. We have tentatively assigned structure G as the most likely, although we cannot rule out H as a possibility.

The N-glucuronide of pyrazole was readily identified as the major component of chromatographic fraction 10. The mass-spectral behavior was identical to that for 4-methyl pyrazole-N-glucuronide (21) and consistent with the structure of I. Three deuterium atoms were retained in this metabolite, as seen in the molecular ion at m/e 532 (535), and the major ions at 204, 217, 305, and 464 (M-68) were characteristic for the glucuronic acid-TMS derivative (21).

There were two additional metabolites of pyrazole, as evidenced by molecular ion isotope doublets at m/e 416 (419) in fraction 3 (fig. 4b) and m/e 504 (506) in fractions 4 and 5. The ions at
Fig. 4. Mass spectra of pyrazole metabolite and synthetic pyrazole riboside.

A, Mass spectrum from fraction 3 (table 1) of a pyrazole metabolite identified as the pyrazole pentose conjugate (TMS). Ion abundances above \( m/e \) 250 are multiplied by 10. B, Mass spectrum of synthetic pyrazole riboside (TMS).

\( m/e \) 147, 189, 217, and 259 were very similar to those observed for the mass spectra of nucleosides (22). The loss of 68 mass units from the molecular ion resulted in the ion at \( m/e \) 348, which also lost the deuterium doublet, and was highly suggestive of structure J, a pentosylpyrazole. This component retained all three deuterium atoms during metabolism. Ribosylpyrazole was synthesized as described in Materials and Methods and trimethylsilylated. The resultant mass spectrum (fig. 4B) differs from the pyrazole metabolite significantly only in the deuterium content of the latter (fig. 4A). The gas-chromatographic behavior of synthetic ribosylpyrazole was identical with that of metabolite J.

The metabolite with the indicated molecular weight of 504 (506) was assigned structure K based on the deuterium doublet pattern (fig. 5), the increase of 88 mass units from metabolite J, and the ions arising from the pentose portion of the molecule. Hydroxylation at the C4 position of the pyrazole nucleus was deduced from the lack of tautomeric hydrogen exchange seen with 3-hydroxypyrazole. It must be stated that the assignment of ribose as the 5-carbon sugar is tentative, inasmuch as other pentoses would display identical mass spectra at this level of analysis.

Discussion

The synthesis of the isotopic variants of pyrazole has provided a means of studying aspects of pyrazole metabolism which have remained unresolved thus far. Studies in the BALB/c mouse and the Sprague-Dawley rat reveal considerable species variation in the disposition of pyrazole. In
neither species was the pyrazole eliminated unchanged. The mouse, as compared to the rat, eliminated radioactivity three times more rapidly and to a greater extent. In neither species, however, was total radioactivity recoverable in urine and feces. Preliminary experiments have indicated that the total elimination of radioactivity is related to the dose of pyrazole. At low doses, most of the radioactivity (>90%) appears in the urine within 4 days.

Structural elucidation of the urinary metabolites revealed a variety of hydroxylated pyrazoles formed in vivo. It is likely that these metabolites have pharmacologic activity of their own and may be responsible for the toxicity of this agent. The N-hydroxy metabolites of aromatic amines have been suspected as being highly toxic as well as having diverse biological effects (23, 24) and pyrazole is converted into at least one N-hydroxy compound, metabolite G.

Identification of pyrazole riboside was totally unexpected. Whereas several drugs, such as 6-mercaptopurine, 5-fluorouracil, and allopurinol are known to be converted to ribosides or ribotides, these agents act as substrates in the salvage pathway for purines and pyrimidines, permitting reutilization for synthesis of nucleotides and nucleic acids. In spite of the fact that allopurinol contains the pyrazole nucleus, it has not been appreciated that pyrazole itself can apparently serve as a substrate for these reactions. One could speculate that the antitumor activity of pyrazole (7, 8) can be a consequence of this route of metabolism. The blockage of hormonal induction of tryptophan oxygenase after pyrazole administration (4) might also be explained by pyrazole ribosides acting as analogs of purine or pyrimidine ribosides and thereby blocking protein synthesis.

Different routes of metabolism may account for the well documented differences in toxicity between pyrazole and 4-methylpyrazole (4MP), in spite of the rather modest structural differences between the two molecules. Similar metabolites of pyrazole and 4MP are the N-glucuronides (21). Diekmann and Garbe (25) reported the N-glucuronide of the pyrazole moiety in a psychoactive pyrazine, and suggested that this structure had unusually high stability toward hydrolysis. There are striking differences between the other metabolites of pyrazole and 4MP. Metabolism of 4MP is centered around oxidation of the methyl group to 4-hydroxymethyl- and 4-carboxypyrazole. The lack of this methyl substituent allows ring hydroxylation, N-oxidation, and ribosylation to be expressed as metabolic products of the parent pyrazole. One or several of these pyrazole metabolites must have properties accounting for the observed toxicity of pyrazole. The methyl group in 4-methylpyrazole not only
increases the affinity of 4-MP for alcohol dehydrogenase, but would also appear to direct metabolism to nontoxic products.

A main thrust of our work has been directed toward the use of stable isotopes and mass spectrometry in pharmacological investigations. The use of stable isotope-labeled drugs in metabolism studies has been reported numerous times [for example, Knapp et al. (19)]. Another advantage of this technique on which we have capitalized has not been previously demonstrated; this is the structural information revealed by the metabolism at or next to a labeled position. The completely deuterated pyrazole (aside from the rapidly exchangeable NH) clearly demonstrated the presence of pyrazole metabolites by the isotope doublets. In addition, the number of deuterium atoms remaining in the metabolite (the second molecular ion at M+1, M+2, M+3) indicated the number of carbon atoms hydroxylated and the position of hydroxylation, which could be used for complete structure elucidation. Each insertion of oxygen on the aromatic ring resulted in a concomitant loss of deuterium atom, and those metabolites that would readily undergo tautomerization displayed an additional loss of the adjacent deuterium atom. This is a further extension of the use of isotopic variants which could have wide application, particularly in drug metabolism studies.

Acknowledgments. Computer programming assistance by H. Russell Helbig is gratefully acknowledged.

References
24. R. Nery, ibid., p. 27.