PHARMACOLOGY OF PYRAZOLES
I: STRUCTURE ELUCIDATION OF METABOLITES OF 4-METHYLPYRAZOLE

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SUMMARY

The metabolism in mice of 4-methylpyrazole (4-MP), a potent inhibitor of alcohol dehydrogenase, has been investigated using gas chromatography and gas chromatography-mass spectrometry techniques. Radioactive 4-MP was synthesized to aid in the isolation of the metabolites. Of the administered radioactivity, 84% was recovered in the urine 24 hours after treatment. Analysis of the urine revealed the presence of several metabolites including 4-hydroxymethylpyrazole, 4-carboxypyrazole and 4-methylpyrazole-N-glucosiduronic acid, along with the parent compound, 4-methylpyrazole.

I. INTRODUCTION

Hepatic alcohol dehydrogenase (ADH, E.C. 1.1.1.1) has been considered a major catalyst in the metabolism of endogenous substrates [e.g., retinol (1), steroids (2), ethanol (3)], as well as a significant participant in the metabolism of alcohols of toxicologic or pharmacologic significance [e.g., ethanol (4), methanol (5), ethylene glycol (6), and hydroxylated drugs (7)]. Pyrazole and certain 4-substituted derivatives exhibit a high degree of inhibitory specificity for purified ADH (1) as well as pronounced inhibitory actions on the oxidation of alcohols in intact animals (8). These characteristics have been employed to advantage in the laboratory (9) and clinic (10) in studying the role of ADH in the metabolism of various alcohol and aldehyde substrates. Unfortunately, interpretation of the results from these studies in vivo has been complicated by a lack of information concerning the disposition of the pyrazoles and the biochemical effects of their metabolites. In this paper we report the results of initial experiments designed to define the major metabolites of 4-methylpyrazole (4-MP), the only pyrazole derivative administered to man in studies of ADH activity (10).

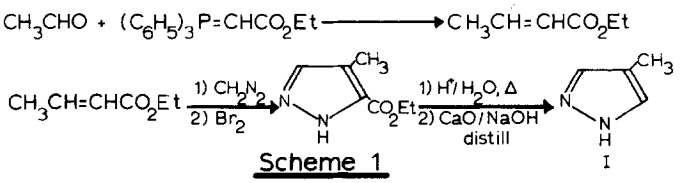
II. TECHNIQUES

Radioactive 4-MP was synthesized from uniformly labeled ^{14}C-acetaldehyde

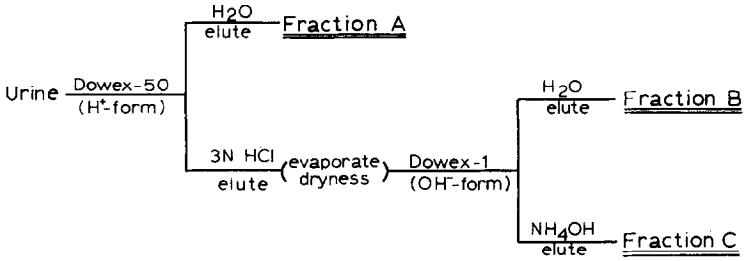
using procedures described previously (11,12) and outlined in Scheme 1. The final product (I) was purified by distillation and was identical to authentic 4-methylpyrazole as determined by its gas chromatographic retention time and mass spectrum.

Male BALB mice (a generous gift of Dr. G. McClearn, Institute of Behavioral Genetics, University of Colorado) were injected intraperitoneally with 2.5 mmole(205 mg)/kg of radioactive 4-MP (0.5 μCi, Specific Activity = 4.93×10^4 dpm/mg) dissolved in 0.9% NaCl solution. Animals were placed in a glass metabolism chamber designed for collection of urine, feces and expired air. Carbon dioxide was collected from the chamber by drawing the effluent air through a series of glass traps containing 3 N NaOH solution.

Urinary metabolites were separated by ion exchange chromatography according to the procedure diagrammed in Scheme 2. Aliquots of the 3 fractions thus ob-



Scheme 1



Scheme 2

tained were assayed for ^{14}C-radioactivity by liquid scintillation spectrometry. Components of these fractions were identified structurally by analysis of data obtained by direct probe mass spectroscopy (MS) of the underiva-

tized compounds, and gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS) of the derivatized compounds. Trimethylsilyl derivatives (TMS) were prepared by reaction with bis(trimethylsilyl)trifluoroacetamide (BSTFA) according to the method of Gehrke (13) and by the procedure described by Hara and Matsushima (14). Gas chromatography of the derivatized metabolites was performed on glass columns packed with 1% OV-7 on Gas Chromosorb-W, while 4-MP was determined on a 5% Carbowax column (15).

III. RESULTS

As seen in Figure 1, 84% of the radioactivity administered as ^{14}C-4-MP was recovered in urine 24 hours after treatment. Radioactivity collected from feces appeared to be a result of urinary contamination. No radioactivity was

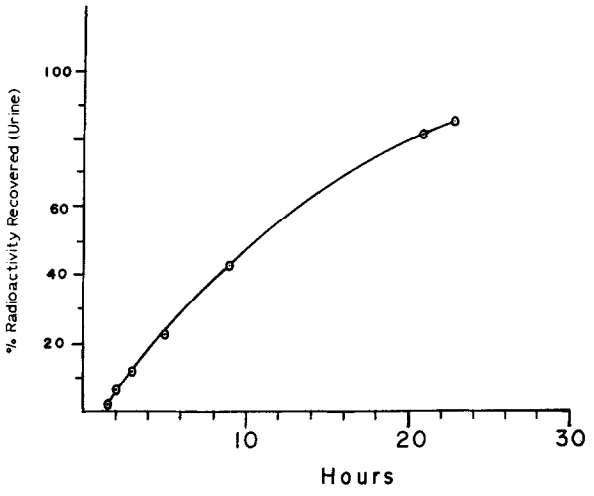
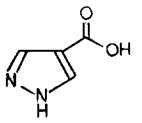


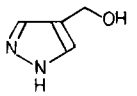
Fig. 1: Accumulated recovery of radioactivity (^{14}C-cpm) in the urine of mice; expressed as the percent of administered ^{14}C-4-methylpyrazole.

detectable in the expired gases during this interval. Urine collected between 5 and 9 hours after administration of 4-MP contained 25% of the total administered radioactivity and was selected for subsequent analysis for metabolites. When urine was extracted by the procedure outlined in Scheme 2,

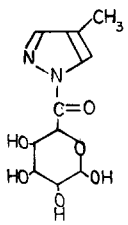
the following distribution of radioactivty was measured: Fraction A (acidic and neutral metabolites), 49.3%; Fraction B (basic metabolites), 45.7%; Fraction C (amphoteric metabolites), 5.0%.



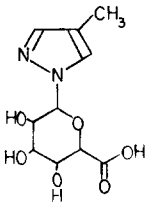
II



III



IV



V

Direct probe mass spectrometry of the components of Fraction C produced intense signals at m/e 112 and 95, suggestive of 4-carboxypyrazole (II), while analysis of Fraction B indicated the presence of 4-hydroxymethylpyrazole (III) from abundant ions at m/e 98, 97, 81.

After trimethylsilylation of Fractions A and B, mass spectra were obtained by GC-MS and the components of these fractions yielded spectra for trimethylsilylated II and III, respectively. Authentic II and III were synthesized from 4-MP (16). The mass spectra and GC retention times of these standards were identical with the metabolites obtained from urine extracts A and B, thus establishing their structural assignments.

All urinary extracts were analyzed for 4-MP, but were measurable only in Fraction B in an amount less than one-half the total radioactivity extracted in this fraction. Fraction A contained the greatest proportion of radioactivity extracted from urine. The GC separation of this fraction after trimethylsilylation is diagrammed in Figure 2. The compound labeled A1 in this

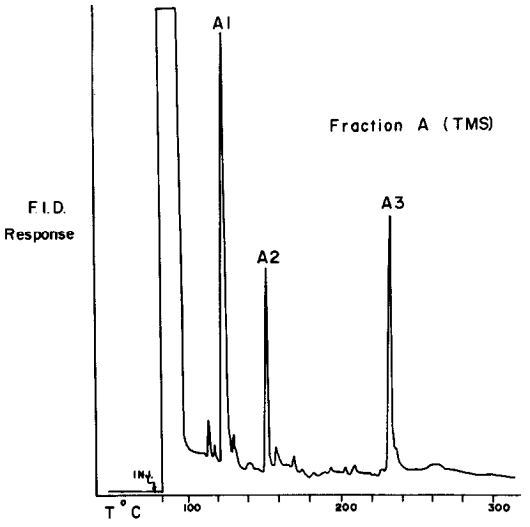


Fig. 2: Gas chromatogram of an aliquot of Fraction A which had been derivatized with BSTFA. Chromatographic conditions: 6 ft x 2 mm I. D. glass column with 1% OV-7 on Chromosorb W. Temperature programmed from 80° to 300° at 16°/min with N_2 carrier gas flow at 30 ml/min and flame ionization detector (F.I.D.).

Figure was identified as tris(trimethylsilyl)phosphate (M^+,m/e 314; M-15, m/e 299 base peak), originally inorganic phosphate in the mouse urine. The mass spectra from A2 were identical to that obtained with trimethylsilyl-4-carboxypyrazole. The mass spectrum recorded during elution of peak A3 (Fig. 3a), the most abundant component in this mixture, is shown in Figure 3b. The intense signals at m/e 204, 217 and 305 were indicative of a carbohydrate or poly-ol derivative (17), and the ion at m/e 531 suggested a molecular weight of 546. A significant ion was recorded at m/e 464, which represents a loss of 82 mass units from the molecular ion, suggestive of the 4-methyl-pyrazole moiety (M.W. = 82). Mass spectral considerations led to the proposal of IV or V as possible structures for this metabolite.

Although no biochemical evidence exists for structure IV, a further experiment was devised in order to provide the information necessary to distinguish the two isomers, IV and V. An aliquot of Fraction A was dissolved

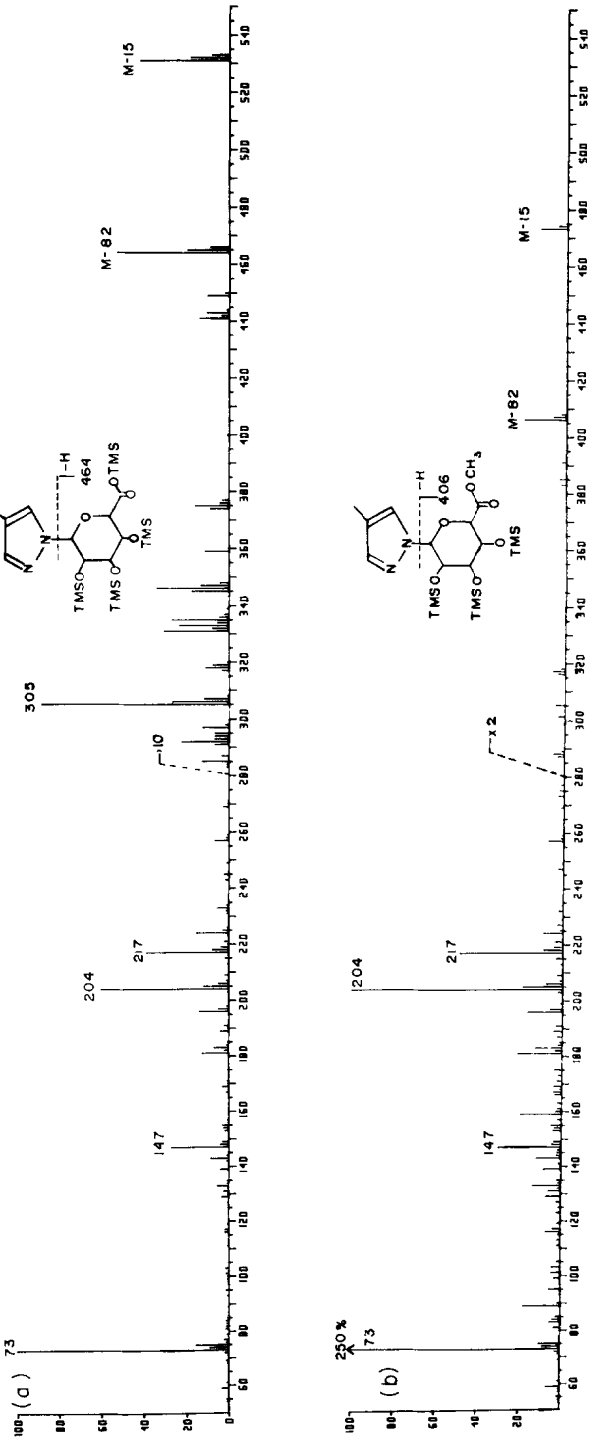


Fig. 3: a) Mass spectrum obtained with the GC-MS-computer system and recorded during the elution of component A3 (Fig. 2). The ion abundances above m/e 280 are multiplied by 10.
b) Mass spectrum of the component in Fig. 3a after initial derivatization of an aliquot of Fraction A with CH_2N_2 followed by trimethylsilylation. The ion abundances above m/e 280 are multipled by 2.

in ethanol and reacted with ethereal CH_2N_2, a treatment which would methylate the carboxylic acid in V but not the anomeric hydroxyl in IV. The solution was then evaporated to dryness, and derivatized by the method of Hara (14), which simultaneously acetylates free amines and trimethylsilylates alcohols. If structure IV were correct, the two different derivatization procedures would produce the same product (tetrakis(trimethylsilyl)ether derivative), and therefore identical mass spectra. In contrast, if structure V were correct, the derivatization procedure would produce a tris(trimethylsilyl)methyl ester and the mass spectrum would therefore show a shift (net loss) of 58 mass units in molecular weight of the compound of interest.

The mass spectrum of component A3 derivatized with CH_2N_2 is shown in Figure 3b. The high mass region (above m/e 300) indicates a shift of 58 mass units for all the significant ions (531 → 473 and 464 → 406). In light of this evidence, compound V appears to be the predominant urinary product formed under these conditions in the BALB mouse.

IV. DISCUSSION

The rationale for the use of the BALB mouse in this study was based on evidence which suggests that this strain terminates the inhibitory action of pyrazole at a rate greater than rats (18) and some other strains of mice (23). It was reasoned that identification of metabolites of 4-MP could be achieved most successfully by employing those animals with the greatest tendency to metabolize this inhibitor.

N-Glucuronic conjugates of aromatic amino compounds, presumably N-glucosiduronic acids, have been recognized for years (19). Boyland (20) presented infrared evidence that the NH-C$\lesssim$ structure occurred in the glucuronide conjugate of 2-napthylamine, however little additional structural determination of intact N-glucuronides has been reported. The structure of the metabolite, 4-methylpyrazole-N-glucosiduronic acid (IV), is consistent with a nucleophilic attack of 4-methylpyrazole on the anomeric carbon of uridine diphosphate glucuronic acid. However, Bridges and Williams (21) suggested that

some N-glucuronides can be formed non-enzymically. Further studies are under way to investigate N-glucuronidation as a pathway in the disposition of 4-MP. Isolation of metabolites may have resulted in the hydrolysis of relatively unstable metabolites, since strong ion exchange resins and concentrated acid were employed in this procedure. Therefore, the occurrence of conjugates of II and III as well as other metabolites is likely. Nevertheless, the identification of 4-hydroxymethylpyrazole and 4-carboxypyrazole demonstrates oxidation *in vivo* of the methyl group of 4-MP to an alcohol and acid. This is of particular interest considering that 4-hydroxymethylpyrazole may also influence ADH activity or other enzymes which catalyze the metabolism of alcohols *in vivo*. Studies are currently in progress to examine this interesting possibility. It is anticipated that studies of this nature, combined with studies on the metabolism of pyrazole, may help to explain the differences in mammalian toxicity of 4-MP and pyrazole (10), species differences in toxicity to pyrazole (9), and species and compound differences in the inhibition of alcohol oxidation *in vivo* (10,22).

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