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Metabolism of Drugs. LXX.[1] Further Study on Antipyrine Metabolism

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In the previous paper,[5] it was shown that antipyrine was oxidized not only to 4-hydroxyantipyrine, but also to 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one which was characterized on the basis of analytical and spectral data.

The present study established the latter pathway, the oxidation of methyl group at 3-position of pyrazolinone ring by the chemical synthesis of authentic sample, and also demonstrated chromatographically the subsequent pathway to 2-methyl-1-phenyl-5-oxo-3-pyrazolin-3-carboxylic acid. Possible importance of this serial oxidation steps was discussed concerning with antipyrine allergy problem.

By the preliminary test it was shown that 3-hydroxymethyl metabolite did not retain the antipyretic acitivity.

Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) has been recognized to be one of the allergenic drugs and this allergy has remained a clinical problem. It is also a generally accepted hypothesis[3] that such a drug of low molecular weight as antipyrine is nonimmunogenic itself, but must first combine irreversibly with a tissue macromolecule (usually a protein) in order to elicit antibody formation which in turn cause allergic reaction. Antipyrine, however, is negligibly bound to plasma proteins and can be used to measure total body water.[4] This fact strongly suggests that allergenicity of antipyrine may associate more directly with its metabolite [s] hitherto unknown.

In the previous paper[5] of this series, the authors reported that antipyrine was metabolized in rats, mice, guinea pigs, rabbits and men, mainly through two oxidation pathways; one was the well known oxidation to 4-hydroxyantipyrine and the other to 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AN–CH_2OH) which was isolated for the first time from the urine of rats and characterized on the basis of elementary analysis and spectral data of ultraviolet (UV), infrared (IR) and nuclear magnetic resonance (NMR). A primary alcohol group of the latter metabolite might be oxidized *in vivo* to an aldehyde capable of reaction with proteins, and further to a carboxyl group.

In order to approach to the drug allergy problem, the present study was undertaken to learn whether or not above serial oxidation steps are involved in the antipyrine metabolism. The experiments concerning with confirmation of the structure of 3-hydroxymethyl metabolite of antipyrine by chemical synthesis and with preliminary examination of antipyretic activity of the same metabolite were also performed.

Material and Method

Antipyrine, mp 111—113°, was obtained from a commercial source. 4-Hydroxyantipyrine, mp 182—183°, was synthesized according to the method described by Knorr and Pshorr.[6]

1) Part LXIX: K. Oguri, S. Îda, H. Yoshimura and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **18**, 2414 (1970).
2) Location: *Katakasu, Fukuoka.*
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4) R. Soberman, B.B. Brodie, B.B. Levy, J. Axelrod, V. Hollander and J.M. Steele, *J. Biol. Chem.*, **155**, 519 (1944).
5) H. Yoshimura, H. Shimeno and H. Tsukamoto, *Biochem. Pharmacol.*, **17**, 1511 (1968).
6) L. Knorr and P. Pshorr, *Ann. Chem.*, **293**, 50 (1896).

Synthesis of the Proposed Metabolites——1) 3-Hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AN-CH_2OH): This compound was synthesized from antipyrine according to the following reaction steps; antipyrine $\xrightarrow{Br_2}$ 4-bromoantipyrine,[7] mp 110—113° $\xrightarrow{NBS}$ 1-phenyl-2-methyl-3-bromomethyl-4-bromo-3-pyrazolin-5-one,[8] mp 135—136° $\xrightarrow{Na_2CO_3}$ 1-phenyl-2-methyl-3-hydroxymethyl-4-bromo-3-pyrazolin-5-one,[8] mp 184—185° $\xrightarrow{H_2/\text{Raney Ni}}$ 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one, mp 144—145°. UV $\lambda_{max}^{0.1N\ NaOH}$ mμ: 243, 260. IR ν_{max}^{KBr} cm^{-1}: 3215 (OH), 1625, 1590. NMR ($CDCl_3$) ppm: 3.15 (3H, singlet, N–CH_3), 4.44 (2H, singlet, CH_2OH), 4.65 (1H, broad peak, CH_2OH), 5.40 (1H, singlet, =C–H), 7.23—7.50 (5H, aromatic). *Anal.* Calcd. for $C_{11}H_{12}O_2N_2$: C, 64.69; H, 5.92: N, 13.72. Found: C, 64.90; H, 5.98; N, 13.92.

2) 3-Formyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AN-CHO): According to the method described by Ito,[9] AN-CH_2OH was oxidized by active manganese dioxide under cooling with water to obtain formyl derivative. It was recrystallized from benzene–ligroin to yellow prisms, mp 118—120° (decomp.). UV λ_{max}^{EtOH} mμ: 244, 275. IR ν_{max}^{KBr} cm^{-1}: 1680, 1660 (CO). NMR ($CDCl_3$) ppm: 3.39 (3H, singlet, N–CH_3), 6.28 (1H, singlet, =C–H), 7.25—7.58 (5H, aromatic), 9.77 (1H, singlet, CHO). *Anal.* Calcd. for $C_{11}H_{10}O_2N_2$: C, 65.33; H, 4.98; N, 13.86. Found: C, 65.60; H, 5.18; N, 13.73.

3) 2-Methyl-1-phenyl-5-oxo-3-pyrazolin-3-carboxylic Acid (AN-COOH): Method 1: Ethyl 1-phenyl-5-oxo-3-pyrazolin-3-carboxylate, mp 89° (10.6 g),[10] which was prepared by condensation of phenylhydrazine and ethyloxaloacetate, was methylated with dimethylsulfate (8.4 g) on a boiling water bath for 1 hr. To the reaction mixture was added 10 ml of water. It was made alkaline with 10% Na_2CO_3, and extracted with AcOEt. The extract was washed with 10% Na_2CO_3, dried over anhyd. Na_2SO_4, and the solvent was evaporated to dryness under reduced pressure. The residue was submitted to silica gel (70 g) column chromatography using 1 liter of benzene–AcOEt (1:1) and 200 ml of MeOH–AcOEt (5:95) as the effluent solvents. From the fraction eluted with benzene–AcOEt, 6.7 g of ethyl 2-methyl-1-phenyl-5-oxo-3-pyrazolin-3-carboxylate was obtained as colorless crystals, which was recrystallized from AcOEt–ligroin to give colorless prisms, mp 87—89°. The mp was identical with that reported previously[11] (mp 86°), and spectral data also supported this structure. IR ν_{max}^{KBr} cm^{-1}: 1745 (ester), 1680. NMR ($CDCl_3$) ppm: 1.30—1.55 (3H, triplet, –CH_2–CH_3), 3.42 (3H, singlet, N–CH_3), 4.25—4.60 (2H, quartet, –CH_2–CH_3), 6.22 (1H, singlet, =C–H), 7.25—7.58 (5H, aromatic).

This ester was then converted to carboxylic acid by usual alkaline hydrolysis. The product was recrystallized from 50% EtOH to afford colorless prisms, mp 200—205° (decomp.). The mp was identical with that reported previously (mp 200°).[11] UV λ_{max}^{EtOH} mμ: 247, 300. IR ν_{max}^{KBr} cm^{-1}: 1710 (COOH). *Anal.* Calcd. for $C_{11}H_{10}O_3N_2$: C, 60.54; H, 4.62; N, 12.84. Found: C, 60.76; H, 4.75; N, 12.85.

Method 2: AN-CHO described above was oxidized with $KMnO_4$ in an usual manner. The product was recrystallized from 50% EtOH to yield colorless prisms, mp 200—207° (decomp.). This carboxylic acid was shown to be identical in details with that prepared by method 1.

4) 2-Methyl-1-phenyl-3-pyrazolin-5-one (M–P–P): AN-COOH described above was decarboxylated by heating at 190—200° in a silicone oil bath, and the resulting oily product was purified by silica gel chromatography using $CHCl_3$, $CHCl_3$–MeOH (99:1) and $CHCl_3$–MeOH (97:3) as the effluent solvents. The fraction eluted with $CHCl_3$–MeOH (99:1) was recrystallized from AcOEt to give colorless prisms, mp 115—117° (reported mp[11]: 117°). IR ν_{max}^{KBr} cm^{-1}: 1645 (CO). NMR ($CDCl_3$) ppm: 3.15 (3H, singlet, N–CH_3), 5.57—5.62 (1H, doublet, =CH–CO), 7.25—7.70 (6H, multiplet, aromatic and =CH–N).

Administration of the Compounds——Antipyrine was dissolved in water and injected *s.c.* to male rats (SD strain) weighing 60—95 g in a dose of 300 mg/kg. AN-CH_2OH dissolved in water was injected *s.c.* to male rats (SD strain) weighing 250—300 g in a dose of 150 mg/kg. After medication, animals were housed in metabolic cages and 48 hr urines were pooled for excretion of the metabolites.

Extraction of the Metabolites——The pooled urine sample was extracted continuously for 9 hr with $CHCl_3$ at pH 2.0 (fraction A), and then at pH 6.6 (fraction B). The urine was finally adjusted to pH 4.6 with AcOH, and to this solution 0.5M acetate buffer, pH 4.6 (20 ml/100 ml of urine) was added. The mixture was incubated with bovine liver β-glucuronidase (activity: 7000 *p*-nitrophenylglucuronide units/ml, 6 ml was added to 100 ml of urine) at 38° for 40 hr. The hydrolyzed urine was extracted continuously with $CHCl_3$ for 9 hr at pH 2.0 (fraction C), and then at pH 6.6 (fraction D). From above four fractions, the solvent was evaporated to dryness under reduced pressure after drying over anhyd. Na_2SO_4, and the resulting residues were used for detection of the metabolites. The urine collected before the medication was used as a negative control.

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8) H. Graef, J. Ledurt and G. Combes, *Bull. Soc. Chim. Belges*, **61**, 331 (1953) [*C.A.*, **47**, 12363 (1953)].
9) I. Ito, *Yakugaku Zasshi*, **76**, 822 (1956).
10) W. Wislicenus, *Ann. Chem.*, **246**, 306 (1888).
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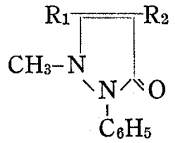
Thin-Layer Chromatography——Thin-layer chromatography was carried out on thin-layer plates, 0.25 mm thick (Silica gel G, Merck), which were activated at 110° for 30 min. The solvent systems used were 1) $CHCl_3$–EtOH (9:1), 2) $CHCl_3$–AcOH–acetone (5:1.4:4), 3) $CHCl_3$–acetone–MeOH (4:4:2) and 4) benzene–AcOEt–EtOH (4:5:1).

The chromatograms were visualized by spraying with Dragendorff, $FeCl_3$ and Ehrlich reagents. Antipyrine and its metabolites give usually orange, brown and red colors, respectively, with the above three reagents. A little heating is necessary, however, to produce color with the Ehrlich reagent. 4-Hydroxyantipyrine does not give a color with either $FeCl_3$ or Ehrlich reagent, but does produce a color with Dragendorff reagent. AN-COOH gives a slightly red color by spraying with Ehrlich reagent followed by heating, and this color turns to a purple color after about 10 hr. This coloration is characteristic to AN-COOH.

The *Rf* values of antipyrine and its proposed metabolites in various solvent systems are shown in Table I.

Table I. The *Rf* Values of Antipyrine and Its Proposed Metabolites in Various Solvent Systems



	4-OH-Anti-pyrine	Anti-pyrine	M-P-P	AN-CHO	AN-CH_2OH	AN-COOH
R_1	CH_3	CH_3	H	CHO	CH_2OH	COOH
R_2	OH	H	H	H	H	H
Solv. system 1	0.55	0.48	0.43	0.35	0.22	0.00
Solv. system 2	0.78	0.58	0.51	0.57	0.38	0.26
Solv. system 3					0.46	0.10
Solv. system 4	0.45	0.22		0.24	0.12	0.00

See text for solvent system.

Result

Identification of 3-Hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AN-CH_2OH), a New Metabolite of Antipyrine

As reported previously,[5] a new metabolite, mp 143—144°, was isolated from the urine of rats as one of the major metabolites of antipyrine and was characterized to be 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one by the elementary analysis and spectral measurements (UV, IR and NMR).

In the present study, the structure of this metabolite was further examined by comparison of the UV, IR and NMR spectra and *Rf* values of thin-layer chromatography in various solvent systems with those of chemically synthesized authentic sample which was described under Methods. Both metabolite and authentic sample were identical in details. The identity was also proved by taking a mixed melting point test.

Detection of the Urinary Metabolites of Antipyrine in Rats

By preliminary examination using an authentic sample of 3-carboxylic acid derivative of antipyrine (AN-COOH), it was proved that the extraction procedure adopted in previous study[5] was not adequate for this carboxylic acid, and therefore antipyrine metabolism in rats was reinvestigated applying a modified procedure which was described under Methods.

Two acidic fractions (A: 312 mg and C: 51 mg) and two neutral fractions (B: 29 mg and D: 14 mg), which were obtained from the pooled urine samples of seven rats given antipyrine (total dose: 166 mg) by extraction at pH 2.0 and pH 6.6 before and after β-glucuronidase treatment, respectively, were submitted to thin-layer chromatography to detect the metabolites. The chromatograms of each fraction are shown in Fig. 1 and 2.

It was clearly shown in these chromatograms that fraction A contained a small amount of AN-COOH, a hitherto unreported metabolite of antipyrine, in addition to unchanged

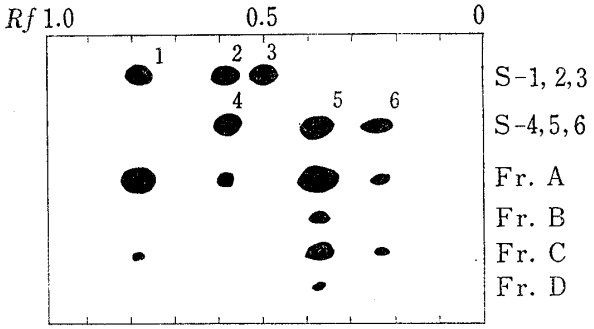


Fig. 1. Thin-Layer Chromatogram of the Urine Extracts of Rats Dosing Antipyrine (Solvent System 2)

S-1: 4-hydroxyantipyrine, S-2, antipyrine,
S-3: M-P-P, S-4: AN-CHO,
S-5: AN-CH_2OH, S-6: AN-COOH

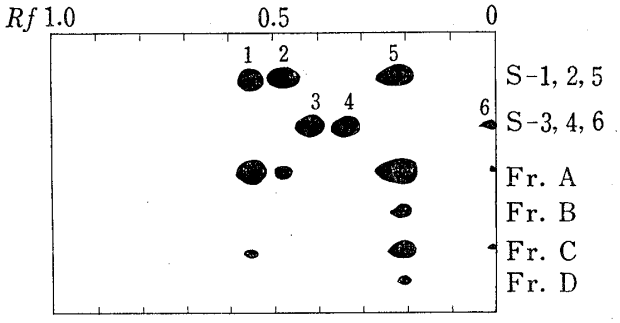


Fig. 2. Thin-Layer Chromatogram of the Urine Extracts of Rats Dosing Antipyrine (Solvent System 1)

S-1: 4-hydroxyantipyrine, S-2: Antipyrine,
S-3: M-P-P, S-4: AN-CHO,
S-5: AN-CH_2OH, S-6: AN-COOH

antipyrine, 4-hydroxyantipyrine and AN-CH_2OH which were described in the previous paper.[5] Most of these free metabolites seemed to be extracted into fraction A, only a part of AN-CH_2OH being further extracted into fraction B. It was also evident from the chromaotgrams of fraction C and D that a detectable amount of above metabolites (4-hydroxyantipyrine, AN-CH_2OH and AN-COOH) was extracted as the corresponding conjugates, too. Existence of AN-COOH on fractions A and C was confirmed not only by the *Rf* values of thin-layer chromatography using several solvent systems, but also by the characteristic coloration with Ehrlich reagent.

As seen in above chromatograms, other possible metabolites, for instance, AN-CHO or a decarboxylated metabolite (M-P-P) could not be detected in any fractions.

Detection of the Urinary Metabolites of AN-CH_2OH in Rats

The metabolites in four fractions (A: 490 mg, B: 61 mg, C: 50 mg and D: 18 mg) obtained from 48 hr urine samples of seven rats (total dose: 205 mg) were characterized by means of thin-layer chromatography. A typical chromatogram using the solvent system 2, is shown in Fig. 3.

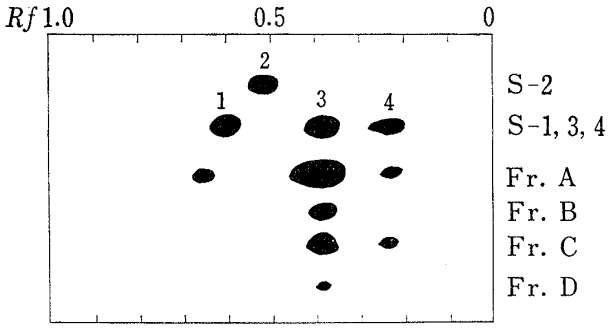


Fig. 3. Thin-Layer Chromatogram of the Urine Extracts of Rats Dosing AN-CH_2OH (Solvent System 2)

S-1: AN-CHO, S-2: M-P-P,
S-3: AN-CH_2OH, S-4: AN-COOH

As judged from this chromatogram, it could be considered that a great part of AN-CH_2OH administered was excreted unchanged, however, a little part was undoubtedly oxidized to AN-COOH. Both AN-CH_2OH and AN-COOH seemed to be partly excreted as the corresponding glucuronides from the chromatograms of fractions C and D.

A spot (*Rf* 0.65) on the chromatogram (solvent system 2) of fraction A, showed the colorations same to those of AN-CH_2OH with three color reagents described under Methods, and therefore seemed very likely as one of the metabolites. The structure of this metabolite, however, remained uncertain.

The proposed metabolites, M-P-P and AN-CHO, could not be detected.

Preliminary Examination of Antipyretic Activity of AN-CH_2OH

Since AN-CH_2OH was found to be a major metabolite of antipyrine in several mammalian species including men, it seemed worthwhile to examine preliminarily whether this metabolite

still retained antipyretic activity comparable to that of antipyrine using male rabbits weighing 2.1—2.5 kg. Rabbits fixed on board were injected intravenously 2,4-dinitrophenol (10 mg/kg) as pyrogen, and at the time when the body temperature was raised 0.5—0.75° at least (30—90 min after the injection), antipyrine (100 mg/kg) or AN-CH_2OH (150 mg/kg) was given intravenously. As the result, about 90 min after injection of antipyrine, the body temperature was lowered 0.4°, but such an effect could not be observed with AN-CH_2OH.

Discussion

In the previous study,[5] a hitherto unreported metabolite was isolated as one of the major metabolites from the urine of rats administered antipyrine and was characterized to be 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AN-CH_2OH). The structure of this metabolite was undoubtedly established in the present study by the comparison with a synthetic sample.

This hydroxymethyl metabolite might further be oxidized to 3-formyl (AN-CHO) and then 3-carboxyl (AN-COOH) derivatives. Antipyrine is not immunogenic itself but an aldehyde derivative would be one of the most noteworthy intermediate capable of reaction with macromolecules in tissues. Such an example can be seen in the study on penicillin allergy.[12,13] Reinvestigation of metabolism of antipyrine of AN-CH_2OH was thus performed, and there was provided the chromatographic evidence that AN-COOH was excreted into the urine as a minor metabolite of both antipyrine and AN-CH_2OH.

Although an aldehyde intermediate could not be detected in the urine of rats given either antipyrine or AN-CH_2OH, the urinary excretion of AN-COOH strongly indicated the possible occurrence of this aldehyde *in vivo*.

The presently demonstrated metabolic pathways of antipyrine in rats are illustrated in Chart 1.

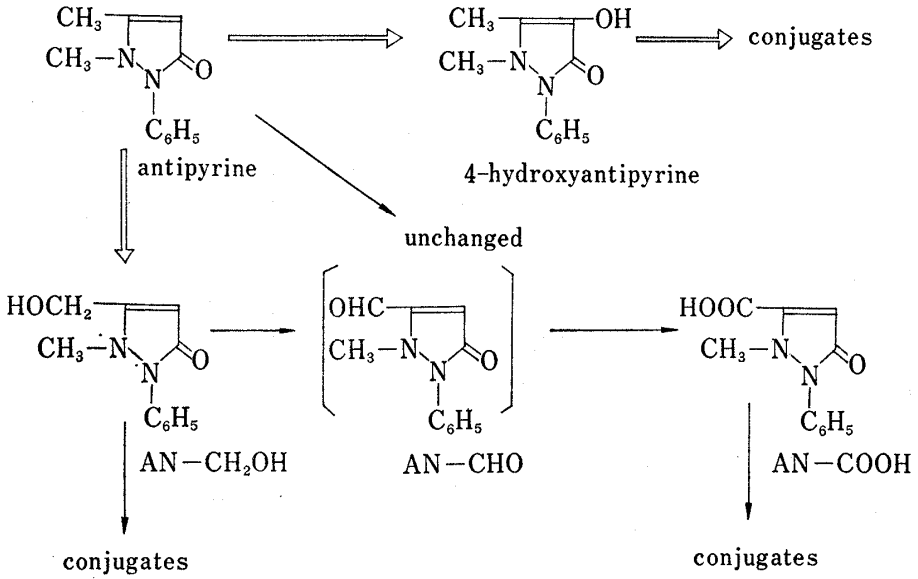


Chart 1. The Demonstrated Metabolic Pathways of Antipyrine in Rats

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They also thank the members of the analytical group of this university for the elementary analysis.

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