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Identification of the Decomposition Products of Hydralazine Hydrazones with Three Endogenous Ketones and Kinetic Study of the Formation of 3-Methyl-*s*-triazolo[3,4-*a*]phthalazine from Hydralazine and Pyruvic Acid[1]

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The decomposition products of hydrazones of hydralazine with pyruvic acid, acetone and α-ketoglutaric acid were studied. The pyruvic acid hydrazone and acetone or α-ketoglutaric acid hydrazone were converted to 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP) and phthalazinone, respectively, both of which are known hydralazine metabolites. New high pressure liquid chromatographic (HPLC) methods for determining hydralazine and MTP were developed. The formation of MTP and the disappearance of hydralazine at a physiological concentration ratio and pH at 37° were studied by the HPLC methods. The formation of MTP followed second-order kinetics, and its rate constant was determined.

Keywords——hydralazine; endogenous ketones; hydralazine hydrazones; 3-methyl-*s*-triazolo[3,4-*a*]phthalazine; phthalazinone; decomposition; formation; identification; kinetic study; high pressure liquid chromatography

Hydralazine has been used to treat essential hypertension by direct peripheral vasodilation. Recently, it has been reported that hydralazine forms hydrazones in the blood with endogenous ketones such as pyruvic acid, acetone and α-ketoglutaric acid after its administration.[3–5] These hydrazones will be called pyruvate, acetonide and α-ketoglutarate hydrazones, respectively, for convenience. We have carried out *in vitro* kinetic studies on the reaction of hydralazine with the three ketones at pH 7.4 and 37°. It was found that the three hydrazones decomposed after their formation. These reactions may also take place in the blood, and might be important from a pharmacological point of view. Accordingly the decomposition products of these hydrazones were examined and identified.

Experimental

Materials——Hydralazine hydrochloride (Tokyo Kasei Kogyo Co., Tokyo) was recrystallized from methanol. Phenytoin of J.P. IX grade, supplied by Dainippon Pharmaceutical Co., Osaka, was used as received. All solvents were distilled before use, and other chemicals were of reagent grade. Decomposition products of hydrazones were separated and purified on silica gel 60G (for TLC, Merck) and aluminum oxide $60GF_{254}$ (for TLC, Merck). Redistilled water was used throughout this study.

Synthesis of 3-Methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP)——A modification of the reported method[6] was used to synthesize MTP. A mixture of 1 g of hydralazine hydrochloride, 0.7 ml of triethylamine and

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10 ml of acetic anhydride was refluxed for 2 hr, and then evaporated to dryness *in vacuo*. The residue was dissolved in chloroform and washed in turn with dil. HCl, 0.1 N NaOH solution, and water. The chloroform layer was evaporated to dryness *in vacuo*. The residue was purified by the preparative TLC method described below in connection with the isolation and purification of the decomposition product of pyruvate hydrazone. The total yield was 300 mg or 32%. The purified product was identified by spectroscopic analyses, *i.e.* NMR (JEOL FX-100) and MS (JEOL JMS-D300), and elemental analysis. MS: found *m/e* 184, $C_{10}H_8N_4$ requires *m/e* 184 (M^+). *Anal.* Calcd for $C_{10}H_8N_4$: C, 65.21; H, 4.37; N, 30.42. Found: C, 64.96; H, 4.34; N, 30.59.

Isolation and Purification of Decomposition Products of Hydrazones——An aqueous solution containing hydralazine hydrochloride and each endogenous ketone was adjusted to pH 7.4 and kept at 37° in the dark for several weeks. Decomposition products were extracted with chloroform or ethyl acetate.

The decomposition products of acetonide and α-ketoglutarate hydrazones were applied to silica gel TLC plates and developed with chloroform–ethyl acetate (2: 1 v/v). A zone at $Rf=0.5$ was scraped off and extracted with acetone. The extract was evaporated to dryness *in vacuo*, and the residue was applied to an aluminum oxide TLC plate and developed with acetone–ethyl acetate (2: 1 v/v). A zone at $Rf=0.2$ was extracted with acetone. The extract was evaporated to dryness *in vacuo*. The colorless residue was a pure substance.

The decomposition product of pyruvate hydrazone was applied to a silica gel TLC plate and developed with acetone. A zone at $Rf=0.4$ was extracted with acetone. The extract was evaporated down and the residue was developed on a silica gel plate with benzene–ethanol (9: 1 v/v). A zone at $Rf=0.3$ was scraped off and extracted with acetone. The extract was evaporated to dryness *in vacuo*. The colorless residue was a pure substance.

Assay of Hydralazine by HPLC——An HPLC method was developed by modifying a GLC assay procedure[7] in which hydralazine is converted into tetrazolo[5,1-*a*]phthalazine. One-half milliliter of 6 N HCl was added to 1 ml of a sample solution, and 0.1 ml of a 50% aqueous sodium nitrite solution was added. The solution was mixed and left to react for 20 min at 0°. The derivative was then extracted with 1 ml of chloroform by shaking for 5 min. After centrifugation, 0.5 ml of the organic phase was removed and evaporated to dryness. The sample tubes were refrigerated untill HPLC analysis was carried out. The dry residue was dissolved in 100 to 200 μl of a phenytoin solution (internal standard) in acetonitrile, and 2 μl of this solution was injected into an HPLC column.

A Jasco TRI ROTAR high pressure liquid chromatograph with a variable wavelength UV spectrophotometer (Jasco UVIDEC-100) and a recorder (Hitachi model 056) was employed. The column was of a reversed-phase type (Jascopak SS-10-ODS-B, 4.6$\phi\times$250 mm). The mobile phase was 35% acetonitrile in redistilled water, and its flow rate was 2.0 ml/min. The UV absorbance at 230 nm, λ_{max} of the tetrazolo derivative, was measured.

Assay of MTP by HPLC——The following method was newly developed. Sodium chloride (0.2 g) was added to 1 ml of a sample solution, and MTP was extracted with 1 ml of chloroform-isopropanol (3: 1, v/v) by shaking for 10 min. After centrifugation, 0.5 ml of the organic layer was removed and evaporated to dryness under a dry air stream at 50°. The residues in the sample tubes were refrigerated until HPLC was carried out. The dry residue was dissolved in 100 to 500 μl of phenytoin solution (internal standard) in acetonitrile and 10 μl of this solution was injected. The mobile phase was 42% acetonitrile in redistilled water and its flow rate was 2.0 ml/min. The UV absorbance at 240 nm, λ_{max} of MTP, was measured.

Disappearance of Hydralazine and Formation of MTP at pH 7.4 and 37°——If it is considered that the maximum plasma level of hydralazine is 500 ng/ml,[8] the concentration ratio of pyruvic acid to hydralazine in the blood is 22.[9] Therefore, the disappearance of hydralazine and the formation of MTP at pH 7.4 and 37° were examined in the presence of the physiological concentration ratio of pyruvic acid to the drug. Phosphate buffer at an ionic strength of 0.1 was used to maintain the pH at 7.4. Hydralazine and MTP were assayed by the newly developed HPLC methods described above.

Results and Discussion

The isolated decomposition products of hydrazones were analyzed spectroscopically. As shown in Fig. 1, the mass spectrum of the decomposition product of pyruvate hydrazone coincided with that of MTP. The NMR spectrum of the product was identical with that of MTP. Thus, it may be concluded that pyruvate hydrazone was converted into MTP in solution at pH 7.4 and 37° (Chart 1).

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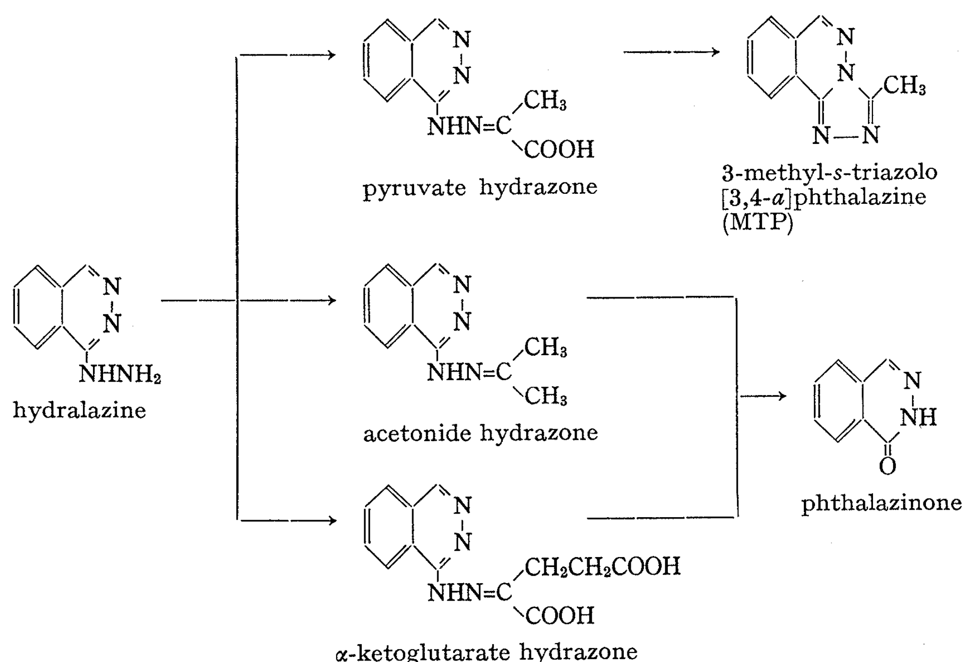

Chart 1. Formation and Decomposition of Hydrazones from Hydralazine and Endogenous Ketones

Table I. Analytical Data for the Isolated Decomposition Product of Acetonide and α-Ketoglutarate Hydrazones

NMR (in $CDCl_3$), δ		MS, m/e
10.1 (1H, bs)[a]	Base peak	146 (M^+)
8.4 (1H, m)		118 (M^+—N_2, or—CO)
8.16 (1H, s)		117 (M^+—N_2, —H·)
7.8 (3H, m)		89 (M^+—N_2, —CO,—H·)

a) Exchangeable. $M^+ + 1$ and $M^+ + 2$ were detected.

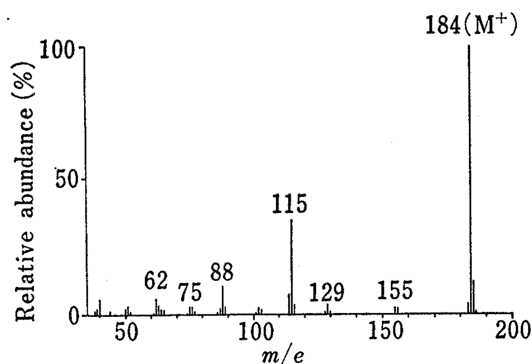

Fig. 1. Mass Spectrum of the Isolated Decomposition Product of Pyruvate Hydrazone

The purified decomposition products of acetonide and α-ketoglutarate hydrazones were identical as judged by HPLC and TLC analyses. Analytical data are presented in Table I. After administration of hydralazine, these hydrazones are expected to form and then to be excreted and/or to decompose in the blood. Therefore, decomposition products of these hydrazones may be present in the blood. Phthalazinone has been identified as a metabolite of hydralazine.[10] The mass spectrum of phthalazinone has been reported[11] and its fragment peaks corresponded with those of the decomposition product. It thus appears that phthalazinone is formed from acetonide and α-ketoglutarate hydrazones (Chart 1).

MTP in blood has been reported to be formed as a result of acetylation of hydralazine,[10] but recently the formation of MTP from hydralazine acetaldehyde hydrazone has been reported.[12] Since the concentration of acetaldehyde in blood is lower than that of pyruvic

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acid,[9] the formation of MTP through acetaldehyde hydrazone should be small. In addition, judging from our kinetic study on these three hydrazones,[13] it is likely that the formation rate of pyruvate hydrazone is larger than that of acetaldehyde hydrazone. Therefore, it may be considered that a part of MTP in the blood is formed from pyruvate hydrazone. Although acetylation polymorphism of hydralazine was reported, it was pointed out that the elimination half-lives of hydralazine among slow acetylators are not significantly different from those among fast acetylators.[8] Thus, enzymatic N-acetylation may not be the major route of metabolism of hydralazine, although further studies are required on this point.

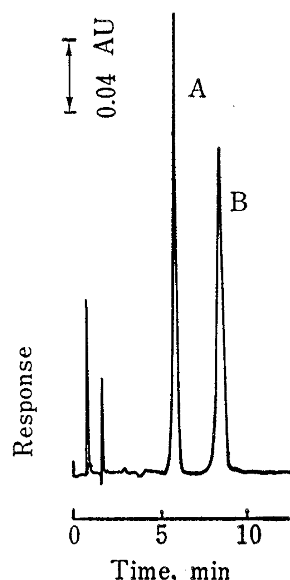

Fig. 2. Chromatogram of a Hydralazine Derivative, Tetrazolo [5,1-*a*]phthalazine (A) and Phenytoin, an Internal Standard (B) Using a Reversed-phase Column

In the GLC assay of hydralazine,[14] acidic conditions were used for derivatization. Under these conditions, however, hydrazones are hydrolyzed to hydralazine, then form tetrazolo-[5,1-*a*]phthalazine with sodium nitrite. Since these three hydrazones have been reported to exhibit the same pharmacological action as hydralazine,[4,5] it seems useful to measure the apparent hydralazine concentration, *i.e.*, the sum of the concentrations of hydralazine and the hydrazones.

A new HPLC assay for hydralazine was thus developed. A typical chromatogram is shown in Fig. 2. In the figure, peak A corresponds to tetrazolophthalazine, which is produced from hydralazine and its pyruvate. A very good straight line was obtained in a calibration plot (r=0.9999). The detection limit was 1 μg/ml in this procedure. In addition, a new HPLC assay for MTP was developed. In the extraction process, pyruvate was not extracted into the organic phase and hydralazine, even if it is extractable to a minor extent, did not interfere with the HPLC assay. Thus, a chromatogram similar to Fig. 1 was obtained. The retention times of phenytoin (an internal standard) and MTP were 4.1 and 6.2 min, respectively. A good straight line was obtained in a calibration plot (r=0.9998). The detection limit of this method was 300 ng/ml in the present procedure. The sensitivity could probably be increased to determine blood levels of hydralazine and MTP.

The disappearance of hydralazine and the formation of MTP at pH 7.4 and 37° were examined, and the results are shown in Fig. 3. The formation of MTP occurred concurrently with the disappearance of hydralazine. The sum of the molar concentrations of hydralazine and MTP was nearly constant over the sampling period. Accordingly, it is likely that most of the hydralazine was converted into MTP through pyruvate hydrazone. A second-order plot for the formation of MTP (Fig. 4) gave a straight line. The formation rate constant was 3.82×10^{-3} mM^{-1} hr^{-1}. Pyruvate hydrazone serves as an intermediate of the second-order reaction in which MTP is formed from hydralazine and pyruvic acid. Because of the high pyruvic acid concentration under the present experimental conditions, the hydralazine concentration decreased apparently following first-order kinetics. The disappearance rate constant was 5.45×10^{-3} hr^{-1}.

In this study, the concentration of hydralazine used was 10 times the drug concentra-

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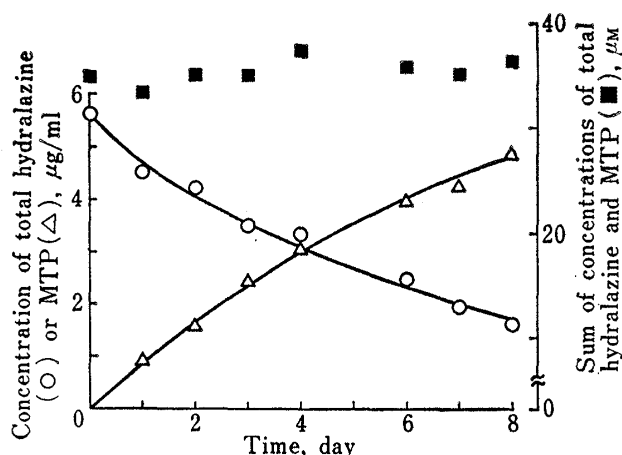

Fig. 3. Time Course of Concentrations of Total Hydralazine (Hydralazine plus its Pyruvate Hydrazone) and MTP in the Presence of a Physiological Concentration Ratio of Pyruvic Acid to Hydralazine (22: 1) at pH 7.4 and 37°

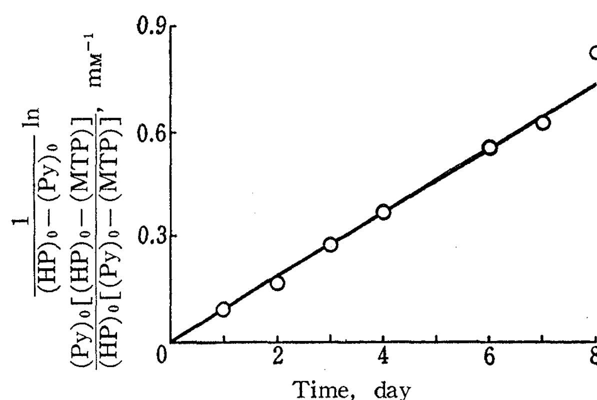

Fig. 4. Second-Order Plot for the Formation of 3-Methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP) from Hydralazine (HP) in the Presence of a Physiological Concentration Ratio of Pyruvic Acid (Py) at pH 7.4 and 37°

tion in the blood. Because the formation of MTP is a second-order reaction, the apparent reaction rate is affected by the concentrations of both hydralazine and pyruvic acid. Thus, it is necessary to study the reaction with drug levels of the same order as that in blood.

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The Effect of Thymosin α_1 Fragments on T-Lymphocyte Transformation in the Uremic State[1]

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The thymosin α_1 C-terminal fragment H-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (positions 14—28) was synthesized by a conventional method. Two thymosin α_1 fragments, H-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH (positions 19—28) and the pentadecapeptide fragment synthesized in this study, were tested for effect on lymphocyte transformation in the uremic state. The synthetic pentadecapeptide increased ^{3}H-thymidine incorporation into DNA in the uremic state, but the synthetic decapeptide had no effect on the ^{3}H-thymidine incorporation.

Keywords——thymosin α_1 fragment; uremic serum; chronic renal failure; HONB-DCC; lymphocytes transformation

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1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: DMF, dimethylformamide; PHA, phytohemagglutinin; WSCI, water-soluble carbodiimide; MA, mixed anhydride; TFA, trifluoroacetic acid; HONB, N-hydroxy-5-norbornene-2,3-dicarboximide; HOBT, N-hydroxybenzotriazole; DCC, dicycloxylcarbodiimide.

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