Chem. Pharm. Bull. 27(11)2820—2826(1979)

UDC 615.225.2.011.5.034.074 : 543.544.2.061

Fate of Hydralazine in Man. I. Reactions under Gastric Conditions[1]

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(Received April 23, 1979)

Tetrazolo[5,1-*a*]phthalazine (Tetra-P) was detected by GC and GC–MS in extracts of incubation mixtures of hydralazine (HP) in human saliva with simulated gastric juice (SGJ), while 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP) and *s*-triazolo[3,4-*a*]phthalazine (Tri-P) were identified as the main products in the absence of SGJ. Tetra-P was also found in rabbit urine when an aqueous solution of $NaNO_2$ was given before and after the oral administration of HP. The amounts of MTP, Tri-P and Tetra-P formed depended on the pH and incubation time.

Keywords——fate of hydralazine; products under simulated gastric conditions; detection by GC and GC–MS; Tetra-P formation in human saliva; formation mechanism of Tetra-P

In the previous paper, we reported the liberation of hydrazine (HZ, a potent mutagen and carcinogen) from hydralazine (HP) in the rabbit and described the mutagenicity of HP itself to *Salmonella typhimurium* TA 100.[3]

When a drug is administered orally, it comes into contact with salivary and gastric components before gastro-intestinal absorption. Even if a drug is given in other dosage forms (*e.g.* injection, enteric tablet, *etc.*), the drug will sometimes reappear in the saliva in the form of intact drug and/or some metabolites after the initial drug absorption. Man secretes about 1.5 l/day of saliva, which contains nitrates and nitrites.[4] Under gastric conditions, not only the formation of nitrosamines but also other chemical transformations could occur through reaction between drugs containing amino moieties and nitrites in the human stomach.

HP has frequently been used for patients with essential hypertension as an effective depressor. Some metabolites of HP have been detected in man and experimental animals.[5] However, no papers have appeared on the anticipated interaction of HP and nitrite to yield tetrazolo[5,1-*a*]phthalazine (Tetra-P) in biological fluids. We examined the fate of HP to explore the possibility of Tetra-P formation in human saliva (*in vitro*) and in rabbit urine (*in vivo*) by means of gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), by which we previously established an accurate and reliable method for determining authentic Tetra-P[6] and other metabolites. The results described in the present paper indicate that the main products formed from HP in human saliva are 3-methyl-*s*-triazolo-[3,4-*a*]phthalazine (MTP), *s*-triazolo[3,4-*a*]phthalazine (Tri-P) and Tetra-P.

1) Presented in part at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978. This work was supported by a Grant-in-Aid for Cancer Research from the Fukuoka Anticancer Association, Japan.
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3) A. Noda, K. Matsuyama, S.-H. Yen, N. Otsuji, S. Iguchi, and H. Noda, *Chem. Pharm. Bull.* (Tokyo), **27**, 1938 (1979).
4) E. Boyland and S.A. Walker, *Nature* (London), **248**, 601 (1974).
5) Z.H. Israili and P.G. Dayton, *Drug Metabolism Reviews*, **6** (2), 283 (1977).
6) J. Drucy and B.H. Ringier, *Helv. Chim. Acta*, **34**, 195 (1951).

Experimental

Chemicals——Reagent grade HP-HCl was purchased from Tokyo Chemical Ind. Co. Ltd., and reagent grade HZ-sulfate from Wako Pure Chemical Ind. Ltd. MTP, Tri-P and Tetra-P were synthesized by the reported methods.[7]

Human Experiment——Twenty ml of mixed saliva was collected from each of four healthy volunteers after fasting for 12 hr.

Animal Experiment——Male albino rabbits weighing between 3.0—3.2 kg were fasted for 16 hr. Drinking water was permitted *ad libitum*. An aqueous solution of $NaNO_2$ (70 mg in 10 ml of water) was given orally by cannulation. One minute later, an aqueous solution of HP-HCl (214.2 mg in 20 ml of water) was administered, followed by a second dose of $NaNO_2$ solution (70 mg in 10 ml of water). Rabbit urine was collected for 24 hr.

Gas Chromatography (GC)——The instrument used was a Shimadzu GC-4CM-PF gas chromatograph equipped with a hydrogen flame ionization detector. A glass column packed with 1.5% OV-17 on Shimalite-W (80—100 mesh) was used for analyses of MTP, Tri-P, Tetra-P and benzalazine (benzylidene derivative of HZ) in all kinds of specimens. In the case of PBH (benzylidene derivative of HP), 1.5% OV-1 was preferred as a packing agent.

Gas Chromatography-Mass Spectrometry (GC-MS)——A JMS-D-100 mass spectrometer coupled to a JGC-20K gas chromatograph was employed. Mass spectrometer conditions were: Accelerating voltage, 3 kV; ionizing current, 300 μA; ionizing energy, 23 eV; separator temperature, 230°.

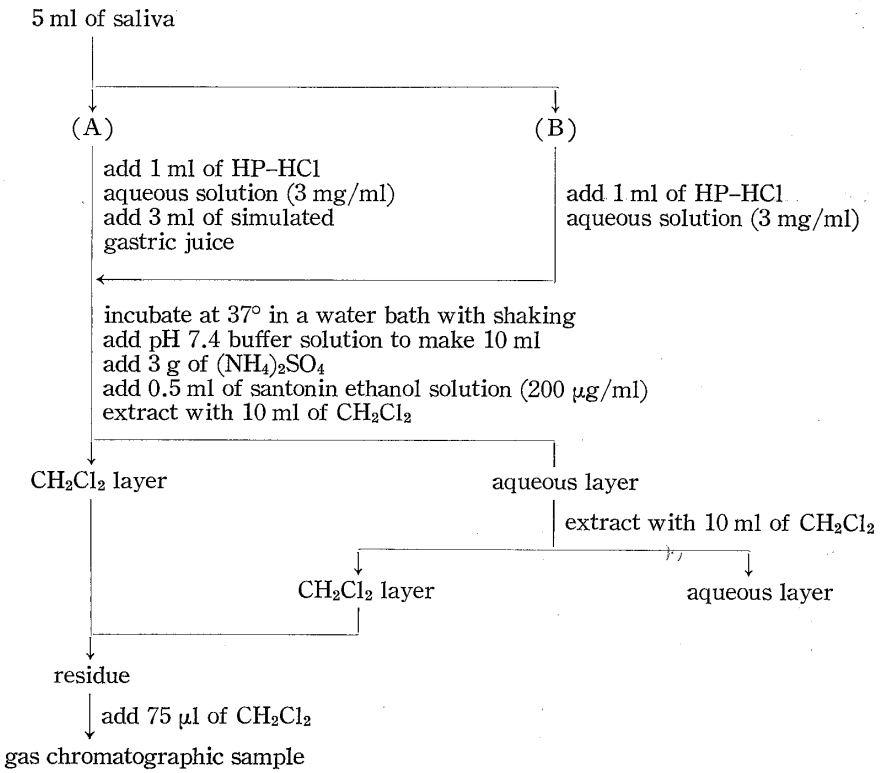

Chart 1. Sample Preparation for Gas Chromatographic Determination of MTP, Tri-P, and Tetra-P

Assay Procedure——(i) Human Saliva: The procedure is shown in Chart 1. First, 1 ml of HP-HCl aqueous solution (3 mg/ml) was added to a 5 ml portion of saliva. The mixed solution was incubated at 37° for 1 hr, then 4 ml of phosphate buffer (pH 7.4) was added. After the addition of 3 g of $(NH_4)_2SO_4$ and 100 μg of santonin (internal standard), the products were extracted twice with 10 ml of CH_2Cl_2. The combined extracts were dehydrated with anhydrous sodium sulfate and evaporated to dryness. The residue was injected into the gas chromatograph as a CH_2Cl_2 solution. Next, 1 ml of HP-HCl aqueous solution (3 mg/ml) was added to another 5 ml of saliva. The mixed solution was incubated at 37° for 1 hr following the addition

7) K.D. Haegel, H.B. Skrtland, N.W. Robie, D. Lalka, and J.L. McNay, Jr., *J, Chromatogr.*, **126**, 517 (1976).

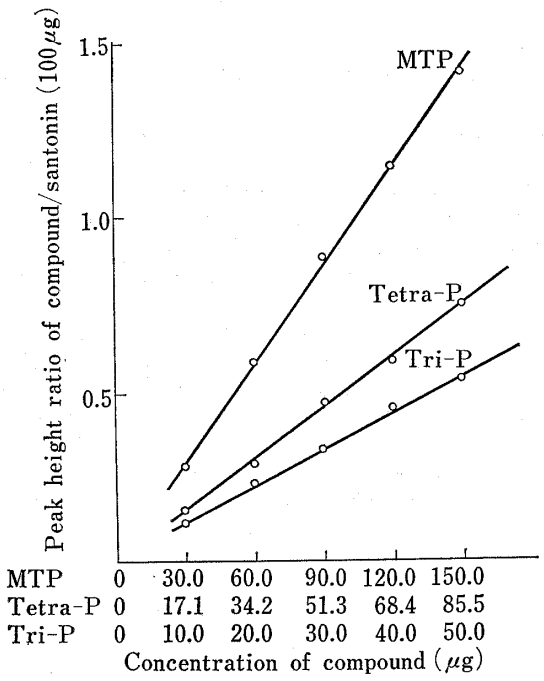

Fig. 1. Calibration Curves for MTP, Tri-P and Tetra-P

*GC conditions are given in the legend to Fig. 3.

of 3 ml of simulated gastric juice (SGJ, VIII Pharmacopoeia, Japan) and 1 ml of phosphate buffer solution (pH 7.4). A GC sample was prepared as described above.

(ii) Rabbit Urine: Rabbit urine samples were extracted by the previously reported procedure.[3]

(iii) Derivatization: In the case of HP and HZ, derivatization with benzaldehyde was necessary prior to analysis.[3]

Calibration Curve——Calibration curves were prepared for MTP, Tetra-P and Tri-P by plotting the peak height ratio of each compound relative to the internal standard (santonin) against the amounts of MTP, Tetra-P and Tri-P added to phosphate buffer solution (pH 7.4), as illustrated in Fig. 1. Linear relationships were obtained in the range of 30.0 to 150.0 μg for MTP, 17.1 to 85.5 μg for Tetra-P and 10.0 to 50.0 μg for Tri-P. A calibration curve was prepared for every run just before the experiment.

Accuracy——The accuracy of gas chromatographic determination of MTP, Tetra-P and Tri-P was studied. Samples of 30.0 μg of MTP, 17.1 μg of Tetra-P, 10.0 μg of Tri-P and 100 μg of internal standard (santonin) were added to 10 ml of phosphate buffer solution (pH 7.4). Each compound was extracted and analyzed as described in "Experimental." The analytical data and recoveries are shown in Table I. As errors due to losses of the compound during extraction, drying and evaporation can be compensated for by the use of the internal standard, most of the error in this determination seems to be attributable to the gas chromatographic analysis procedure.

Table I. Recoveries of MTP, Tetra-P and Tri-P

	Added amount (μg)	Santonin (μg)	Compound/ Santonin	Ratio found	Recovery	Mean ± S.D.
MTP	30.0	100	0.300	0.279	0.93	0.907±0.021
	30.0	100	0.300	0.268	0.89	
	30.0	100	0.300	0.270	0.90	
Tetra-P	17.1	100	0.171	0.140	0.82	0.813±0.050
	17.1	100	0.171	0.130	0.76	
	17.1	100	0.171	0.148	0.86	
Tri-P	10.0	100	0.100	0.116	1.16	1.083±0.068
	10.0	100	0.100	0.103	1.03	
	10.0	100	0.100	0.106	1.06	

Results and Discussion

Saliva was collected from healthy volunteers in our laboratory after fasting for 12 hr. The mixed saliva was divided into two parts and mixed with HP–HCl. Simulated gastric juice (SGJ) was added to one test tube containing the saliva to check the possible reactions of the drug in the stomach. Into another saliva specimen, phosphate buffer solution (pH 7.4) was poured instead of SGJ. The products derived from HP were assayed by GC. Each mass spectrum on GC–MS of the corresponding fractions obtained from the extracts of both reaction mixtures was coincident with that of the appropriate authentic sample (Fig. 2 and Fig. 3). These results indicate that the main products formed from HP were MTP, Tri-P and Tetra-P, and showed that their yields differed markedly in the absence and presence of

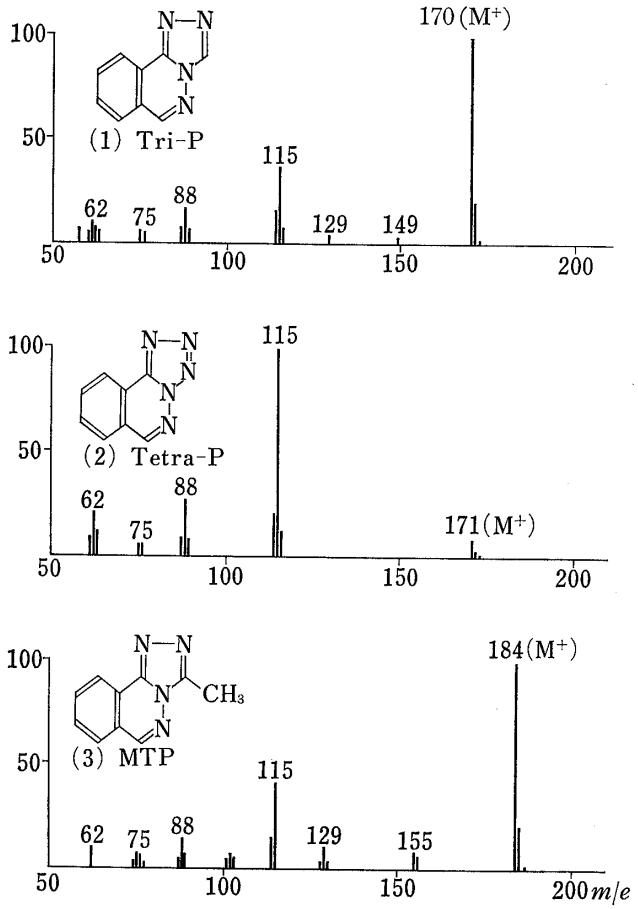

Fig. 2. Mass Spectra of (1) Tri-P, (2) Tetra-P and (3) MTP

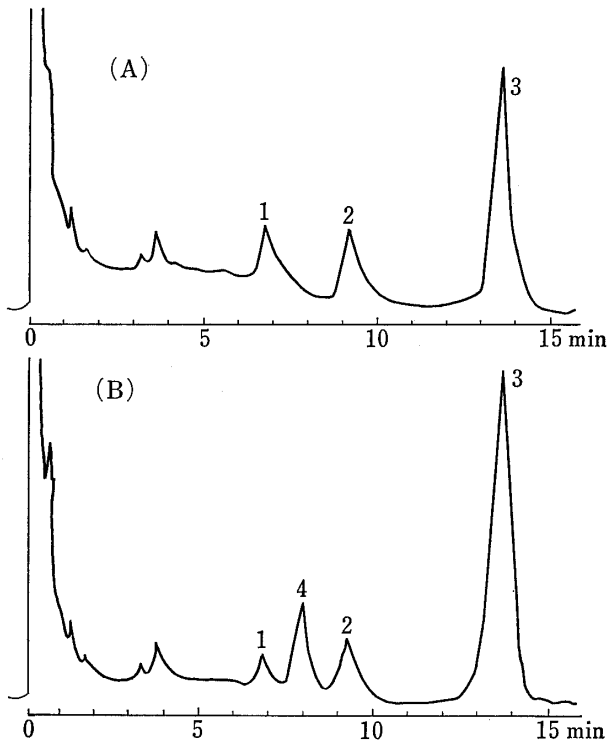

Fig. 3. Gas Chromatograms of Products derived from Hydralazine after Incubation at 37° for 1 hr

GC Conditions:
Column: 1.5% OV-17 on Shimalite-W, 3mm × 2m glass column.
Column temp: 215°. Inj. temp: 250°.
Carrier gas: N_2, 40 ml/min. FID.
(A): human saliva only.
(B): human saliva + SGJ.
1: Tri-P. 2: MTP. 3: santonin (I.S.). 4: Tetra-P

Table II. Transformation of Hydralazine in Human Saliva at 37° (*in vitro*)

Hyaralazine mg	Reaction conditions		Yields of products, μg		
	Medium[a]	Incubation time, hr	MTP	Tri-P	Tetra-P
3	S1	2	28	20	—
		4	32	20	—
	S2	2	78	24	—
		4	127	32	—
40	S3	1	58	15	—
		4	112	22	—
	S4	1	6	6	—
		4	70	20	—
3	S1+SGJ	2	17	9	28
		4	20	8	26
	S2+SGJ	2	29	4	63
		4	28	8	61
40	S3+SGJ	1	36	15	54
		4	40	14	72
	S4+SGJ	1	26	8	32
		4	24	13	34

a) All transformations were carried out in 5 ml of human saliva with or without 3 ml of SGJ. S1, S2 S3 and S4 are salivas collected from volunteers just before the run.

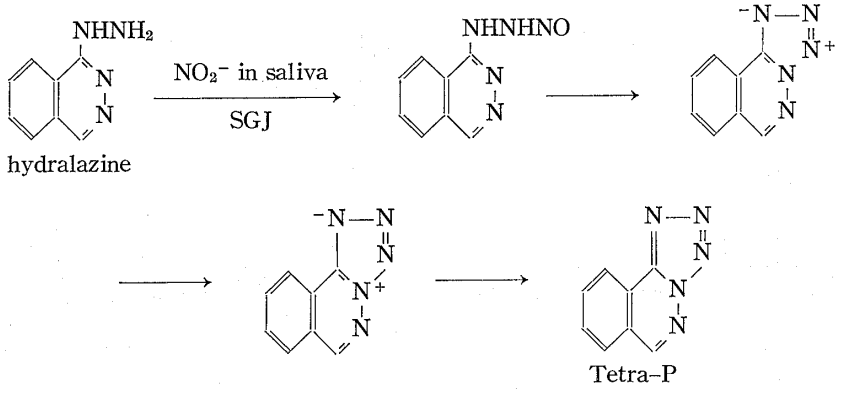

Chart 2. Formation of Tetra-P from Hydralazine in Human Saliva under Gastric Conditions

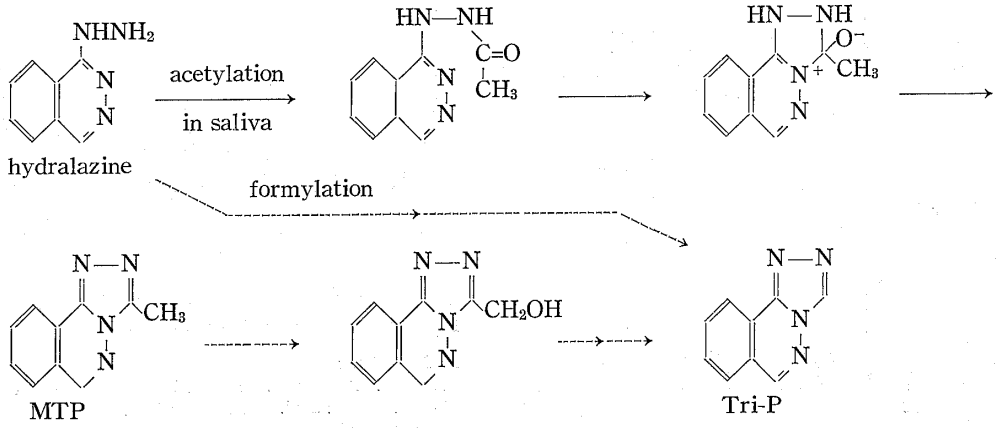

Chart 3. Formation of MTP and Tri-P from Hydralazine in Human Saliva

SGJ, *i.e.*, the formation ratio of the products depends upon the pH of the reaction medium (Fig. 3 and Table II).

Based on the probable mechanisms of formation of Tetra-P (Chart 2), and of MTP and Tri-P (Chart 3), it is reasonable that Tetra-P is the major product in the presence of SGJ or under acidic conditions, while MTP is formed mainly in the absence of SGJ, and Tri-P should be formed either by the oxidation of MTP or by the formylation of HP. In addition, the amount of MTP formed depends not only on pH but also on the incubation time (Table II). Wagner *et al.* reported that about 7% of MTP formed from the administered dose of HP was excreted in human urine.[8] Our results suggest that a part of the metabolite MTP detected in human urine may be a reaction product of HP with a salivary acetylating component.

Although MTP may be formed by the cyclization of an unstable intermediate, acetyl-HP, it is not clear whether such acetylation is carried out by an endogenous enzyme or a bacterial enzyme. Since no report on acetylating components in saliva has been published, we are investigating this problem.

In order to examine Tetra-P formation under gastric conditions, the following experiment was performed in a rabbit. As the nitrite level in rabbit saliva was negligible, an aqueous solution of $NaNO_2$ was given orally to a rabbit before and after the administration of HP–HCl. The 24 hr urine was analyzed by the method reported in the previous paper.[1] The results of GC are illustrated in Figs. 4 and 5. Intact HP and a hydrolysis product HZ were deriva-

8) J. Wagner, J.W. Faigle, P. Imhof, and G. Liehr, *Arznei. Forsch.*, **27**, 2388 (1977).

tized by treatment with benzaldehyde to yield benzalazine (BZ) and phthalazinobenzylidene hydrazine (PBH) prior to GC.[3]

In the presence of $NaNO_2$ solution, Tetra-P was detected as the major metabolite, together with HZ and intact HP (Fig. 4C). Another experiment without $NaNO_2$ administration was performed using the same rabbit after a 1 week rest period. The result was quite different; we could detect MTP, Tri-P, HZ and intact HP, but not Tetra-P (Fig. 4B).

The observation that the Tetra-P formation from HP and $NaNO_2$ *in vitro* is very rapid, especially under acidic conditions, being complete within 1 min in acidic buffer solution (pH

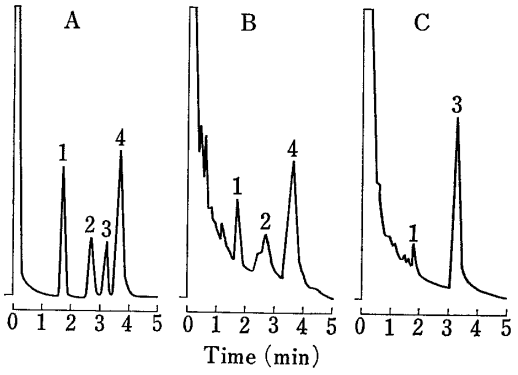

Fig. 4. Gas Chromatograms of Metabolites of Hydralazine

A: authentic samples.
B: metabolites in rabbit urine.
C: metabolites in rabbit urine with administration of $NaNO_2$.
1: benzalazine. 2: Tri-P. 3: Tetra-P. 4: MTP.
GC conditions:
Column: 1.5% OV-17 on Shimalite-W, 3mm × 1m glass column.
Column temp: 220°.
Inj temp: 250°.
Carrier gas: N_2, 40 ml/min.
FID.

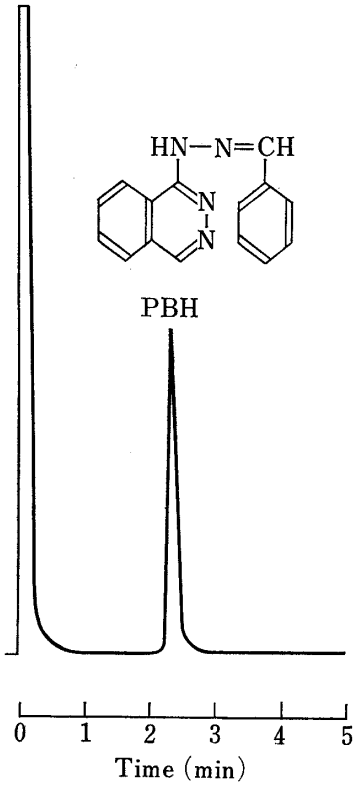

Fig. 5. Gas Chromatogram of Authentic PBH

GC conditions:
Column: 3mm × 1m glass column, 1.5% OV-1 on Shimalite W.
Column temp: 240°.
Inj. temp: 250°.
Carrier gas: N_2, 40 ml/min.
FID.

Table III. Rate of Formation of Tetra-P from Hydralazine Hydrochloride and Sodium Nitrite at 37°

Time (min)	pH 1.1 (0.1 N HCl)	pH 2.1 (0.01 N HCl +0.09 M NaCl)	pH 3.0 (0.05 M AcONa +0.05 M NaCl +0.05 N HCl)
<1	78.7%	73.1%	71.3%
3	74.1%	74.6%	73.0%

The amount of Tetra-p was determined by GC using MTP as an internal standard. Initial concentration of $NaNO_2$, 5.90×10^{-3} M: hydralazine hydrochloride, 5.85×10^{-2} M. As a part of the $NaNO_2$ was decomposed under the conditions described above, a Tetra-P yield of more than about 80% cannot be expected.

1.1, 2.1 and 3.0) at 37°, suggests that Tetra-P formation occurs rather than MTP formation under gastric conditions (Table III).

The GC method established by Jack *et al.*[9] for HP analysis in biological fluids contains a prior reaction with $NaNO_2$ in hydrochloric acid. However, our observation of Tetra-P formation under human gastric conditions indicates that their method may not be suitable for HP analysis in biological fluids, especially in human specimens.

In order to examine Tetra-P and HZ formation, we are now investigating the fate of HP in human patients on HP treatment.

9) D.B. Jack, S. Brechbuhler, P. Degen, P.M. Zwinden, and W. Riess, *J. Chromatogr.*, **115**, 87 (1975).