sides (Rf 0.20 and 0.37)*3; fractions 19~21 contained the nucleoside (IV, Rf 0.20); yield, 566 mg. (39.7%). This sample (Rf 0.20) consumed metaperiodate under a standard condition. UV $\lambda_{max}^{H_2O}$ mμ: 270, 230. IR cm^{-1}: $\nu_{C=O}$ 1720. Though this sample was analytically pure, attempts to crystallize failed. *Anal.* Calcd. for $C_{11}H_{15}O_6N_3$: C, 46.31; H, 5.30; N, 14.73. Found: C, 45.98; H, 5.26; N, 14.58.

Product Distribution of Acetylation of (I) with Glacial AcOH——Cytidine (I, 243 mg., 1 mmole) was treated with glacial AcOH (10 ml.) for 3 hr. at refluxing temperature. The solvent was removed *in vacuo* to leave brown syrup. Chromatography of the reaction mixture showed the presence of 5'-O-acetyl-(Rf 0.20, 57%), and 2' (or 3'), 5'-O-diacetylcytidine (Rf 0.37, 17%) in addition to unreacted cytidine (0.08, 26%). Even after the solution was refluxed for another 3 hr., the product distribution did not change.

N^4,5'-O-Diacetylcytidine (V) from 5'-O-Acetylcytidine (IV)——To a solution of 5'-O-acetylcytidine (IV, 285 mg., 1 mmole) in 50 ml. of pyridine was added 0.1 ml. of Ac_2O. The solution was refluxed for 2 hr. The solvent was removed *in vacuo* to leave gummy residue. Crystallization from EtOH afforded 274 mg., 83.8% yield of N^4,5'-O-diacetylcytidine, m.p. 127~130°. This sample consumed metaperiodate under a standard condition. UV $\lambda_{max}^{H_2O}$ mμ: 248, 296. IR cm^{-1}: $\nu_{C=O}$ 1720, 1730. *Anal.* Calcd. for $C_{13}H_{17}O_7N_3$: C, 47.71; H, 5.23; N, 12.84. Found: C, 47.52; H, 5.35; N, 12.80.

N^4,5'-O-Diacetylcytidine (V) from N^4-Acetylcytidine (II)——To a suspension of N^4-acetylcytidine (II, 2.26 g., 7.9 mmoles) in 150 ml. of dry pyridine was added 0.8 ml. of Ac_2O (*ca.*, 8 mmoles). The suspension was refluxed for 2 hr. The solvent was removed *in vacuo* to leave gummy residue. Crystallization from EtOH afforded a pure sample; yield, 2.12 g. (82%), m.p. 125~129°. UV $\lambda_{max}^{H_2O}$ mμ: 248, 296. IR cm^{-1}: $\nu_{C=O}$ 1720, 1730. Mixed m.p. with the authentic sample described above did not show depression. *Anal.* Calcd. for $C_{13}H_{17}O_7N_3$: C, 47.71; H, 5.23; N, 12.84. Found: C, 47.43; H, 5.49; N, 12.70.

N^4-Benzoylcytidine (III)——To a solution of cytidine (2.43 g., 10 mmoles) in pyridine (70 ml.) and dimethylformamide (30 ml.) was added benzoic anhydride (3.39 g., 15 mmoles). The reaction mixture was stirred for overnight at room temperature, after which it was poured into water and stirred. The solvent was removed *in vacuo* to leave white solid. The solid material was extreacted with ether (four 40 ml. portions) to remove BzOH. The yield of residue was 3.2 g. (92%), m.p. 218°. The analytical sample was prepared by crystallization from EtOH; m.p. 219~220°. UV λ_{max}^{MeOH} mμ: 305, 260. IR cm^{-1}: $\nu_{C=O}$ 1730. *Anal.* Calcd. for $C_{16}H_{17}O_6N_3$: C, 55.04; H, 4.94; N, 12.1. Found: C, 55.05; H, 4.95; N, 12.20.

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*3 Nucleoside (Rf 0.37) was assumed to be 2'(or 3'),5'-O-diacetylcytidine, based on its absorption maxima and the fact that the spot (0.37) showed a negative reaction for a metaperiodate spray reagent and the fact that Rf-values in the same solvent of 2'(3'),5'-O-diacetyl-, 2',3',5'-O-triacetyl-, and N^4,2',3',5'-O-tetraacetylcytidines are 0.37, 0.48 and 0.73, respectively.

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Toshio Imanari and Zenzo Tamura: Gas Chromatography of Compounds in Vitamin B_6 Group.

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A highly sensitive determination method for the compounds in vitamin B_6 group has been requested because of the extremely small quanties of them present in biological fluids. The fluorometric method and bioassay are usually available, but they are troublesome and tedious, and have some defects. The application of gas chromatography should provide a rapid and simple method. In addition, the microdetermination may

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be developed, when trifluoroacetyl (TFA) derivatives are subjected to gas chromatograph equipped with electron capture detector (ECD). For this reason, the preliminary examinations were made on gas chromatographic separation of pyridoxal (PAL), pyridoxine (PIN), pyridoxamine (PAM) and pyridoxic acid lactone (PIA-L) as TFA derivatives.*[2]

Treatment of these compounds with trifluoroacetic anhydride (TFAA) in tetrahydrofuran (THF) caused instant trifluoroacetylation as described below. TFA derivatives of PIN, PAM and PIA-L thus prepared were stable and volatile enough for the gas chromatographic separation, although the derivative of PAL did not give a single peak. The difficulty was overcome through converting PAL to a methyloxime or a Schiff's base prior to trifluoroacetylation; N,N-dimethylhydrazine was also investigated as a specific reagent for the carbonyl group, but the N,N-dimethylhydrazone of PAL was decomposed during trifluoroacetylation.

As shown in Table I, the derivatives, PAL (=$NOCH_3$, TFA), PIN (TFA), PIA-L (TFA), PAM (TFA) and α-naphthylamine (TFA) were resolved completely on DC 550 column (Fig. 1). Moreover, an excellent separation of PAL (Pro, TFA) and other TFA

Table I. Retention Times of TFA Derivatives of B_6 Group Compounds

Stationary Phase & Column temp.	Compound						
	PAL (TFA)	PIN (TFA)	PIA-L (TFA)	PAM (TFA)	PAL (Pro, TFA)	PAL (=$NOCH_3$, TFA)	α-NA[b] (TFA)
2% CNSi 160°	0.99[a]	2.07	3.75	7.59	9.75	2.39	5.48
5% DC 550 140°	1.80[a] (2.08)	2.05	9.00	5.60	—	4.40	10.90

a) The peak of PAL (TFA) gave a broad peak which had a slight shoulder.
b) α-NA: α-naphthylamine, This is suitable for internal standard.

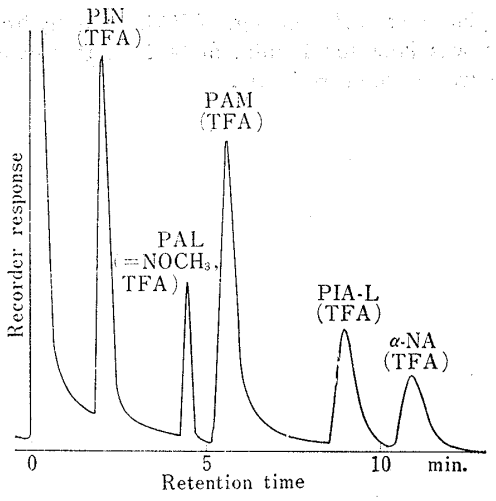

Fig. 1.

Condition: Column, 5% DC550 on Gas-Chrom P, 1.8 m. × 4 mm. i.d.
Temp., Column 140°
Sample Chamber 140°
HFID 160°
Carrier Gas, N_2 90 ml./min.
Sens. 100 Range 0.4 V.

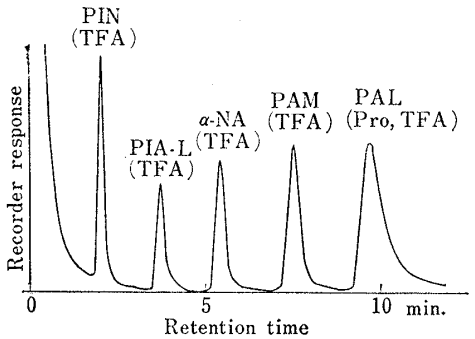

Fig. 2.

Condition: Column, 2% CNSi on Gas-Chrom P, 1.8 m. × 4 mm. i.d.
Temp., Column 160°
Sample Chamber 160°
HFID 180°
Carrier Gas, N_2 70 ml/min.
Sens. 100 Range 0.4 V.

*[2] During this investigation, B. Paul, *et al.* reported the gas chromatography of compounds in the vitamin B_6 group as acetyl and trimethylsilyl derivatives (Anal. Biochem., **17**, 66~75 (1966)).

derivatives of vitamin B_6 analogues were also achieved on CNSi column (Fig. 2).

A typical chromatogram obtained with ECD from a mixture of nanogram order amounts of PIN, PAM and PIA-L is shown in Fig. 3.

High sensitivity and complete resolution promise the application for analysis of the compounds in vitamin B_6 group in biological fluids.

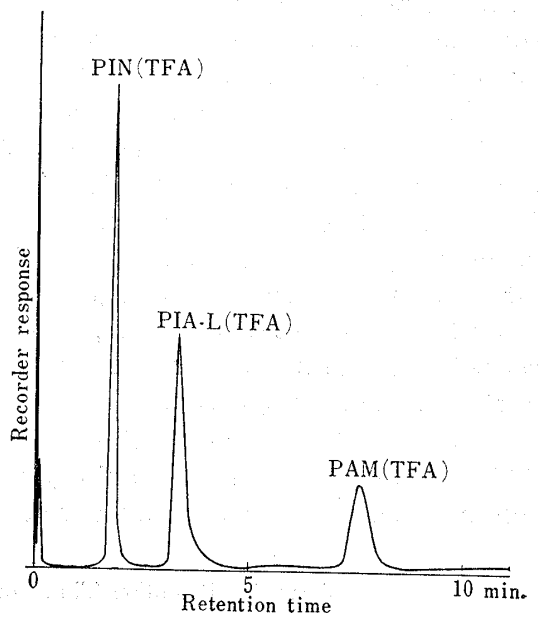

Fig. 3. Gas Chromatographic Separation of TFA Derivatives of PIN (0.1 ng.), PIA-L (2.0 ng.) and PAM (0.2 ng.)

Condition: Column, 2% CNSi on Gas-Chrom P, 1.8 m. × 4 mm. i.d.
Temp., Column 150°
Sample Chamber 150°
ECD 180°
Carrier Gas, N_2 80 ml./min.
Volt. 10 V.
Sens. 100 Range 0.8 V.

Experimental

Materials——Vitamin B_6 analogues used in this experiment were obtained commercially.

Apparatus——Shimadzu Gas Chromatograph Model GC-1C equipped with hydrogen flame ionization detector (HFID) and electron capture detector was used. A glass tube (1.8 m. × 4 mm. i.d.) was packed with 2% CNSi (GE XF 1105) or 5% DC 550 on Gas-Chrom P (80~100 mesh).

Preparation of Derivatives——A) TFA derivatives: To 0.1~0.5 mg. of samples, 0.2 ml. of THF and 0.1 ml. of TFAA were added. The mixture was kept for 10 min. with occasional shaking and 1~2 μl. of the solution was injected directly into the gas chromatograph equipped with HFID.

B) TFA derivative of methyloxime of PAL: 0.1~0.5 mg. of PAL or its hydrochloride and an excess of O-methylhydroxylamine hydrochloride were placed into a ground-glass stoppered test tube and dissolved in 0.2 ml. of dry pyridine. After standing for 10 min. at 50°, the reaction mixture was evaporated to dryness under N_2 stream. The residue was treated as described in A).

C) TFA derivative of Schiff's base of PAL with *n*-propylamine: 0.1~0.5 mg. of PAL or its hydrochloride was dissolved in 0.5 ml. of *n*-propylamine and the solution was kept for 10 min. at 30°. After evaporation of the excess of the reagent under N_2 stream, the residue was treated as in A).