iii) Most polar fraction (135 mg.) was identified with the starting material by mixed melting point and IR spectrum.

3β-Acetoxy-14β-hydroxy-15α-thiocyanato-5β-card-20(22)-enolide (IX)——To 10 ml. of HSCN-$CHCl_3$ solution (34 mg./ml.) 224 mg. of 3β-acetoxy-14β,15β-epoxy-5β-card-20(22)-enolide (Ⅷ) was added and the solution was allowed to stand at room temperature, protected from light, for 4 days. The reaction mixture was washed with H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. The crude product was separated directly into three fractions by preparative TLC (SiO_2, acetone-$CHCl_3$=1 : 5 and toluene-AcOEt=1 : 1) in order to avoid regeneration of the epoxide (Ⅷ) from thiocyanatohydrine.

i) The first fraction (58 mg.) was the starting material (Ⅷ) recovered intact.

ii) The second fraction (109 mg.) was crystallized to 56 mg. of needles of 3β-acetoxy-14β-hydroxy-15α-thiocyanato-5β-card-20(22)-enolide (Ⅸ), m.p. 190~195°. $[\alpha]_D^{24.5}$ +76.5° (c=0.518, $CHCl_3$). *Anal.* Calcd. for $C_{26}H_{35}O_5NS \cdot \frac{1}{2}H_2O$: C, 64.70; H, 7.52; N, 2.90; S, 6.74. Found : C, 64.75; H, 7.40; N, 3.20; S, 6.78. UV λ_{max}^{EtOH} mμ (ε): 215 (17,300). IR ν_{max}^{Nujol} cm^{-1}: 3462(OH), 2160(SCN), 1786, 1746, 1625 (butenolide), 1694 (Ac).

iii) The third fraction (58 mg.) was crystallized to 15 mg. of needles of 3β-acetoxy-15-oxo-5β,14α-card-20(22)-enolide (X), m.p. 225~230°. Mixed melting point and IR spectrum established the identity with an authentic sample of X.

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Summary

Ring-opening reaction of 3β-acetoxy-14α,15α-epoxy-5β-card-20(22)-enolide (Ib) with thiocyanic acid proceeded smoothly to afford 3β-acetoxy-14β-thiocyanato-15α-hydroxy-5β-card-20(22)-enolide (Ⅱ) and 3β-acetoxy-14β-isothiocyanato-15α-hydroxy-5β-card-20(22)-enolide (Ⅲ). On the contrary, the reaction with 3β-acetoxy-14β,15β-epoxy-5β-card-20(22)-enolide (Ⅷ) did not proceed so smoothly as a result of steric hindrance, giving 3β-acetoxy-14β-hydroxy-15α-thiocyanato-5β-card-20(22)-enolide (Ⅸ) together with 3β-acetoxy-15-oxo-5β,14α-card-20(22)-enolide (Ⅹ). Ring closure of the mesylate of Ⅱ with a weak alkali resulted in the formation of 3β-acetoxy-14β,15β-epithio-5β-card-20(22)-enolide (Ⅷb) and 3β-acetoxy-14α,15α-epoxy-5β-card-20(22)-enolide (Ib).

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88. Satoshi Kawai*1, Toshiharu Nagatsu*2, Toshio Imanari, and Zenzo Tamura*1 : Gas Chromatography of Catecholamines and Related Compounds.

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A number of physiologically important amines have been successfully analyzed by gas chromatography. But effective methods for the gas chromatographic analysis of catecholamines are still lacking. This is largely because these amines are strongly polar, nonvolatile, rather insoluble in most organic solvents and extremely unstable.

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The first significant gas chromatographic studies on biological amines were reported by Fales and Pisano[1] who described a method for the separation of many aromatic amines as the free bases on a 4% SE-30 column. Most biological amines have several functional groups, and it is usually necessary to prepare suitable derivatives before attempting a gas chromatographic separation. Sen and McGeer[2] found that suitably volatile derivatives of the catecholamines could be prepared by conversion to trimethylsilyl (TMS) ethers. These compounds showed excellent gas chromatographic properties, but the TMS derivatives of epinephrine and norepinephrine were not separated each other on a 6% SE-30 column nor were the derivatives of metanephrine and normetanephrine. Lindstedt[3] recently reported the separation of the TMS derivatives of dopamine, epinephrine, norepinephrine, metanephrine and normetanephrine using the stationary phase consisted of 0.5% QF-1 mixed with 0.05% ethylene glycol succinate. The use of a thinly coated polar liquid phase, however, such as QF-1 and ethylene glycol succinate, led to the appearance of extensive tails for the highly polar compounds. Horning, *et al.*[4,5] studied a separation of the catecholamines through a formation of acetylated derivatives. The separation of four catecholamine derivatives was achieved with a F-60-Z phase under temperature programmed conditions. They have emphasized that the acetyl derivatives are suitable for the gas chromatographic separation of the catecholamines because of their potential utility in the isolation of amines from dilute aqueous solution, together with their resistance to hydrolysis and their general stability. However, the formation of a fully acetylated product was rather difficult.

On the other hand, Brochmann-Hanssen and Svendsen[6] showed that the products (presumably Schiff's base or oxazolidines) from reaction of ephedrine and related amines with acetone were satisfactory derivatives for gas chromatography. Fales and Pisano[1] found that a primary amine dissolved in acetone exhibited a second peak after the expected amine peak and presumed that this was due to the formation of monomeric Schiff's base between solvent and solute, which resulted in an increase of molecular weight. Later Horning, *et al.*[5] have described work on the separation and identification of tryptamine-related indole bases through a reaction with hexamethyldisilazane (HMDS) in acetone solution to yield the corresponding TMS ether and Schiff's base derivative. In the present work, a method for gas chromatographic separation of catecholamines and related compounds, such as epinephrine, norepinephrine, dopamine, metanephrine and normetanephrine was studied by techniques involving TMS ether formation with HMDS in the pyridine solution, followed by condensation reaction with ketones. Among ketones tested, acetone reacted most readily with the primary amines. For instance, the TMS derivative of dopamine perfectly reacted with a drop of acetone in a few minutes at room temperature. Norepinephrine and normetanephrine needed to stand for 10 to 20 minutes at room temperature, or to be warmed for a few minutes in a 50 to 60° water bath. The completeness of the reaction was followed gas chromatographically in each instance by disappearance of the peak of the TMS derivative on the chromatogram. As would be expected, epinephrine and metanephrine, being the secondary amine, gave no reaction with acetone under the above reaction conditions. A separation of the derivatives produced from the reaction of three catecholamines with HMDS and acetone is illustrated in Fig. 1

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3) S. Lindstedt : Clin. Chim. Acta, **9**, 309 (1964).
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B. A separation of norepinephrine and epinephrine was achieved on an SE-30 column, but it did not distinguish dopamine from epinephrine. By the use of 3-pentanone in place of acetone, the complete separation of three catecholamines, dopamine, epinephrine and norepinephrine, was achieved as shown in Fig. 1C. However, 3-pentanone, probably because of the bulkier ethyl group, reacted incompletely to show a second peak before the expected peak in the case of norepinephrine, which overlapped with epinephrine.

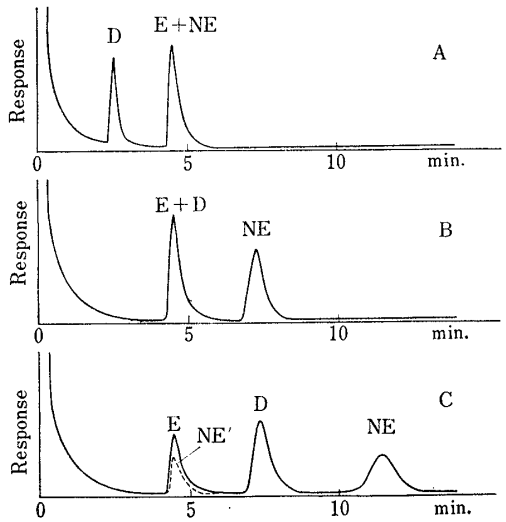

Fig. 1. Separation of Three Catecholamines

D: dopamine
E: epinephrine
NE: norepinephrine
NE': reaction byproduct of NE
Derivatives: trimethylsilyl ether (A); Schiff's base-trimethylsilyl ether (B, acetone condensation products; C, 3-pentanone condensation products)
Conditions: 3.5% SE-30 on 60/80 mesh Gas-Chrom P. 1.5m.×4 mm., 180°

For the elimination of the above problems, 2-pentanone was used, by which a single peak of norepinephrine was produced. A typical chromatogram of a mixture of the catecholamines and related compounds through the reaction with HMDS and 2-pentanone in pyridine was shown in Fig. 2. The ketone derivatives, furthermore, gave nontailing symmetrical peaks that should be suitable for quantitative work,

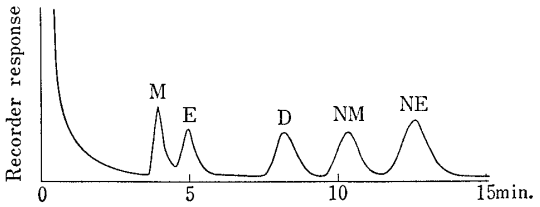

Fig. 2. Separation of Catecholamines and Related Compounds as the Schiff's Base-Trimethylsilyl Ether Derivatives (2-pentanone Condensation Products)

M: metanephrine
E: epinephrine
D: dopamine
NM: normetanephrine
NE: norepinephrine
Conditions: the same as Fig. 1

as evident in Fig. 1C or 2. It is considered that the polarity of amine is masked by the azometine group of the corresponding Schiff's base.

On the trimethylsilylation of the catecholamines, one of the troublesome problems is the fact they are insoluble in most organic solvents. To overcome this difficulty, Sen and McGeer[2] accomplished the trimethylsilylation of them by treatment for 20 hours at room temperature with HMDS in pyridine, while Lindstedt[3] heated the amines to 80° for 5 to 15 minutes in HMDS and dimethylformamide. In the present paper, considering that these amines are extremely unstable and undergo remarkable decompositions by the standing for long time in solution, TMS ethers were prepared by suspending them in pyridine and HMDS, and warming the mixture in a 80 to 90° water bath for about 15 minutes to become transparent. This technique is better in regard to accomplishing the trimethylsilylation for short time. Horning, *et al.*[5] prepared the Schiff's base-TMS derivatives through the reaction with HMDS in acetone. But, when epinephrine suspended in 2-pentanone was heated with HMDS for longer time than 30 minutes for trimethylsilylation, it sometimes resulted in the formation of several byproducts as shown in Fig. 3. Brochmann-Hanssen and Svendsen[6] have described the fact that some of the secondary amines react with ketone, presumably by the oxazolidine formation. 2-Pentanone was therefore added to the reaction solution after the trimethylsilylation of the amines and the mixed solution was warmed again for 10 minutes. The TMS derivative of epinephrine was not affected by these

reaction conditions.

The main problem in gas chromatography is that of finding a suitable phase and derivatives which will provide the necessary separation. The compounds in the study are highly polar and only slightly volatile. An SE-30, which is a nonpolar stationary phase, was suitable for the separation of the highly polar compounds. The use of QF-1 or diethyleneglycol succinate, which is polar liquid phase, did not improve the separation of these derivatives and caused extensive tailing. As to the derivatives of the catecholamines, it was considered that TMS ethers were more suitable than acetylated amined. The catecholamines gave a remarkable decomposition when the temperature of the injection zone was above 300°. It would also be desirable to keep the temperature of injection zone at as low as possible.

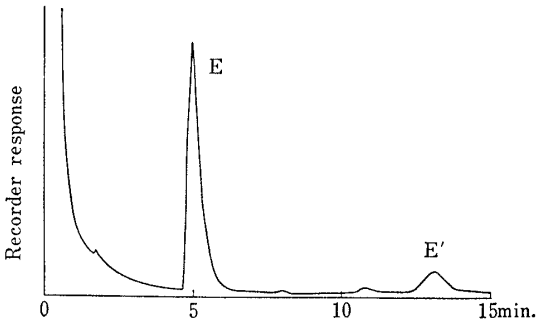

Fig. 3. Gas chromatogram of Epinephrine heated in Pyridine, Hexamethyldisilazane and 2-Pentanone for 1 Hour at 80~90° Water Bath

E: epinephrine
E': reaction byproduct of E
Conditions: the same as Fig. 1

Experimental

Gas Chromatography——A Shimadzu model GC-1B gas chromatograph equipped with flame ionization detector was used. The solid support was Gas-Chrom P (Applied Science Laboratories, State College, Pennsylvania), 60 to 80 mesh, washed first with concentrated hydrochloric acid and then with acetone, and treated with dimethyldichlorosilane in toluene followed by an anhydrous methanol rinse. The solid support was suspended in 3.5% SE-30 siloxane polymer dissolved in toluene and filtered by suction to remove the nonadsorbed phase and solvent. The nearly dry product was dried at 80° overnight and packed in a stainless U tube, 1.5 m. in length × 4 mm. internal diameter which was then ready for use after 6 to 8 hr. of conditioning at 260°. The column and the injection temperature were 180°, and the detector was kept at 200°. A flow rate of 120 ml./min. of the carrier gas (nitrogen) was used.

Derivative Formation——About 0.5 mg. of the amine or its hydrochloride in a 10 ml. glass-stoppered test tube was treated with 0.1 ml. of pyridine and 0.1 ml. of HMDS. The mixture was shaken mechanically for about 10~20 minutes in a 80 to 90° water bath to become transparent and then 0.5 ml. of 2-pentanone was added to the reaction solution. The mixed solution was warmed again for 10 minutes at the same temperature as the above and a 0.5 μl. aliquot could be injected directly into the column without further purification.

Summary

A gas chromatographic method was described employing a 3.5% SE-30 siloxane polymer as the liquid phase which permitted the separation of the catecholamines and related compounds. The satisfactory separation of epinephrine, norepinephrine, dopamine, metanephrine and normetanephrine was achieved through the trimethylsilylation with hexamethyldisilazane followed by the condensation with 2-pentanone.

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