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Studies on the Coloration Mechanism of Furostanol Derivatives with Ehrlich Reagent. I. On the Reaction of 3,26-Dimethoxy-furost-5,20-diene with Ehrlich Reagent[1)]

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From the reddish reaction mixture of 3,26-dimethoxy-furost-5,20-diene (ψ-diosgenin dimethyl ether, **2**) with Ehrlich reagent, a colorless condensation product was isolated and characterized as 3,26-dimethoxy-23-(*p*-dimethylaminobenzylidenyl)-furost-5,20-diene (**3**). However, a coexisting red product could not be isolated because of its instability. On treatment with dried hydrogen chloride or conc. hydrochloric acid, the colorless chloroform solution of **3** readily changed into a reddish solution, which was reversibly decolorized on addition of 3% ammonia–chloroform solution. Based on ultraviolet and carbon-13 nuclear magnetic resonance spectral analysis, the coloration mechanism of **2** with Ehrlich reagent has been inferred to be as follows. The reaction is initiated by condensation between *p*-dimethylaminobenzaldehyde and **2** to form **3**, followed by protonation to form an iminium cation (**3B**) under acidic conditions.

Keywords——Ehrlich reaction; 3,26-dimethoxyfurost-5,20-diene; pseudodiosgenin dimethyl ether; *p*-dimethylaminobenzaldehyde; tautomerism; ^{13}C-NMR

The Ehrlich reaction[2)] has been used for the detection of active methylene or methine groups in such compounds as pyrrole derivatives[3)] and primary amines,[4)] giving a red, purple or yellow coloration. The coloration mechanism of this reaction was investigated with several pyrrole derivatives,[3)] but no detailed report has appeared.

In 1968, one of the authors, Kiyosawa, and co-workers[5)] found a wide distribution of proto-type saponins (22-hydroxy- or alkoxyfurostanol glycosides) in Dioscoriaceous and Liliaceous plants by making use of the red coloration with the Ehrlich reagent. They also found that ψ-steroid sapogenin 26-acetate was positive in the Ehrlich reaction, but a spirostanol derivative was negative. Thus, the active center of furostanol or pseudofurostanol derivatives might be around C_{22}. The present paper describes the coloration mechanism of Ehrlich reagent with a pseudofurostanol derivative, 3,26-dimethoxyfurost-5,20-diene (ψ-diosgenin 3,26-dimethyl ether, **2**), which is assumed to be one of the intermediates in the course of the Ehrlich reaction between a furostanol derivative and *p*-dimethylaminobenzaldehyde under acidic conditions.

ψ-Diosgenin diacetate (**1**), $C_{31}H_{46}O_5$, derived from diosgenin acetate by Marker's degradation method,[6)] was converted into 3,26-dimethoxyfurost-5,20-diene (**2**) to avoid any influence of the hydroxyl groups on the Ehrlich reaction. Ehrlich reaction of **2** with *p*-dimethylaminobenzaldehyde in the presence of hydrochloric acid proceeded smoothly at room temperature. The reddish reaction mixture was purified by repeated Avicel column chromatography to afford colorless needles, $C_{38}H_{54}NO_3$, (**3**), but the red product could not be isolated on account of its instability. The infrared (IR) spectrum (KBr) of **3** showed absorption bands ascribable to an aromatic ring at 1615, 1520, and 810 cm^{-1} and to tertiary amine at 1355 and 1235 cm^{-1}, while the proton nuclear magnetic resonance (^{1}H-NMR)

spectrum revealed the presence of an $-N(CH_3)_2$ group (δ 2.95 ppm, 6H, s), aromatic protons (δ 6.67, 7.23 ppm, each 2H, d, $J=9$ Hz), 18-CH_3 (δ 0.79 ppm, s), 27-CH_3 (δ 0.88 ppm, d, $J=6$ Hz), 19-CH_3 (δ 1.02, s), 21-CH_3 (δ 1.79, s), two O–CH_3 group (δ 3.28, 3.35, each s), 16-CH (δ 4.80, m) and 6-CH (δ 5.32, $W_h=12$ Hz). The ultraviolet (UV) spectrum of **3** showed the absorption maximum at 320 nm, corresponding to that of *p*-dimethylaminobenzaldehyde at 360 nm. Based on the IR and ^{1}H-NMR analyses, a fundamental steroidal skeleton and a partial structure of *p*-dimethylaminobenzaldehyde in **3** were anticipated, and the structure of **3** was finally established by comparing the carbon-13 nuclear magnetic resonance (^{13}C-NMR) of **2**, *p*-dimethylaminobenzaldehyde, and **3** as follows. The ^{13}C-NMR spectrum of **3** showed almost all the carbon signals corresponding to those of **2** and *p*-dimethylaminobenzaldehyde except for the C_{23} carbon signal of **2** at δ 23.4 ppm and the aldehyde carbon signal of *p*-dimethylaminobenzaldehyde at δ 189.9 ppm; these signals were replaced by signals at δ 129.5 (s) and 131.8 (d) ppm assignable to a trisubstituted double bond (this was further supported by the ^{1}H-NMR spectrum, showing a singlet olefinic proton at δ 6.55 ppm). Therefore, the structure of **3** was deduced to be 3,26-dimethoxy-23-(*p*-dimethylaminobenzylidenyl)furost-5,20-diene, formed by dehydration between the aldehyde group of *p*-dimethylaminobenzaldehyde and C_{23} methylene of **2**. The geometrical configuration of the *p*-dimethylaminophenyl group and steroidal skeleton of **3** was assumed to be entirely *cis* based on the ^{13}C-NMR spectra. Lower or higher field shifts by 0.3—2.8 ppm of each carbon signal of the steroidal E ring of **3** were evident, while significant shifts of the side-chain carbon signals of the steroidal moiety were not observed.

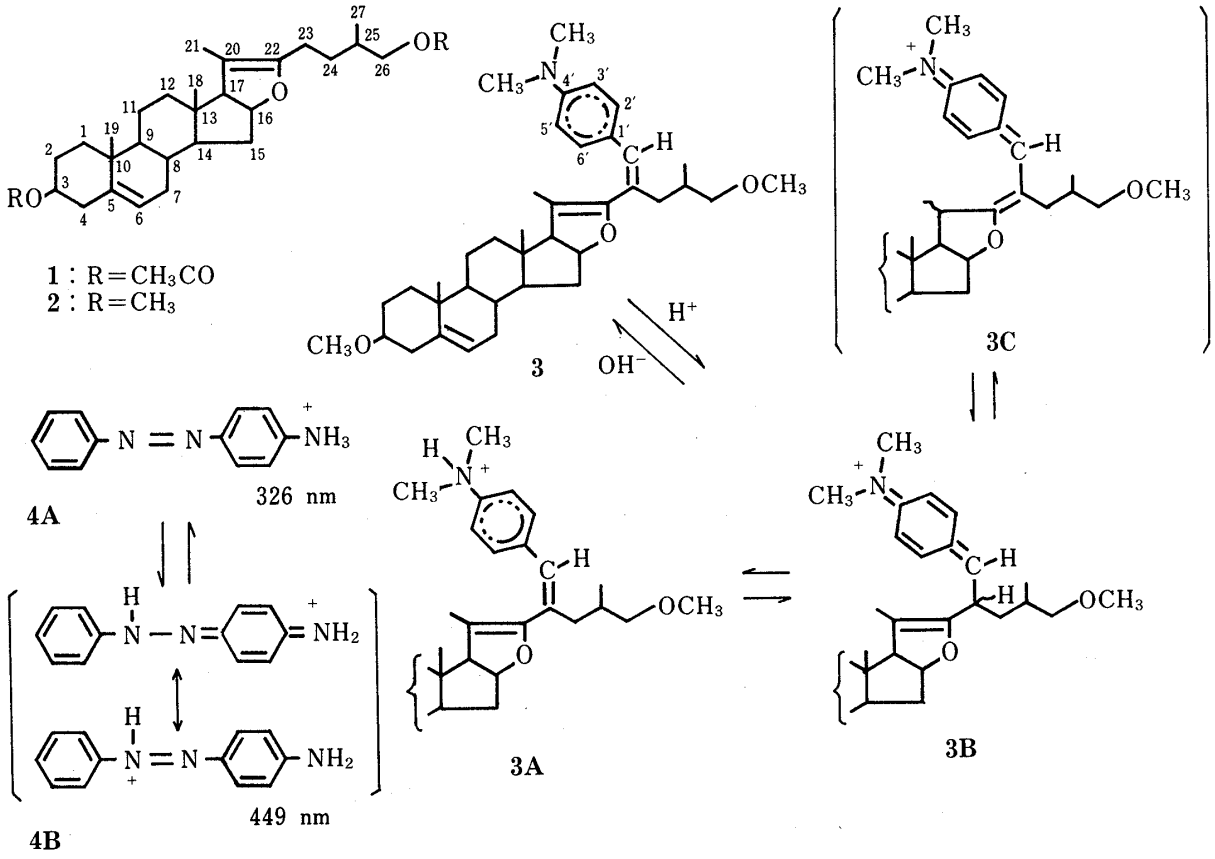

Chart 1

Next, the coloration mechanism of **3** in chloroform solution containing hydrochloric acid was investigated. Recently, Kuroda *et al.*[7] pointed out that 4-aminoazobenzene exists in an acidic medium in two tautomeric forms, an ammonium cation (**4A**) and an iminium cation

(**4B**), which show absorption maxima at 326 nm (**4A**) and 499 nm (**4B**), respectively, in the UV spectra. The time-course of UV absorption of **3** in chloroform solution was recorded over a period of 1 h after addition of hydrochloric acid, and the results are illustrated in Fig. 1. As shown in Fig. 1, the intensity of the 320 nm ($\varepsilon = 20900$) band of **3** in chloroform solution decreased with time after the addition of acid (3% ethanolic hydrochloric acid-chloroform) and the intensities of the newly formed absorption bands at 250 nm (A) and 520 nm (B) increased gradually. A distinct isosbestic point at 392 nm was observed on the absorption curve. When the acid colored solution was slowly neutralized by addition of an equivalent amount of 3% ammonia–chloroform solution, the color and absorption bands of the solution did not change in the course of the neutralization process, but in the last phase of neutralization, bands A and B suddenly decreased and the color of the solution faded immediately to show the absorption maximum at 320 nm. These spectral changes are shown in Fig. 2. The reversible color changes of the chloroform solution of **3** were confirmed to be reproducible.

Furthermore, the coloration mechanism of **2** in acidic chloroform solution was studied

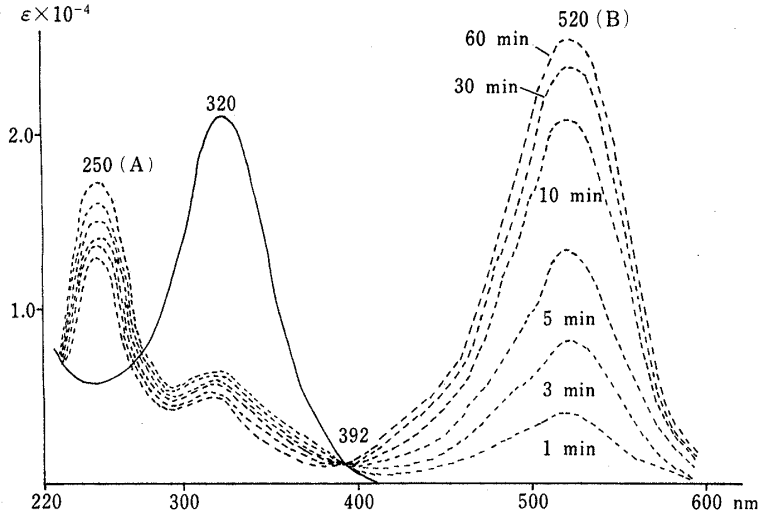

Fig. 1. Absorption Spectra of **3** in Chloroform at Various Times after Addition of Acid

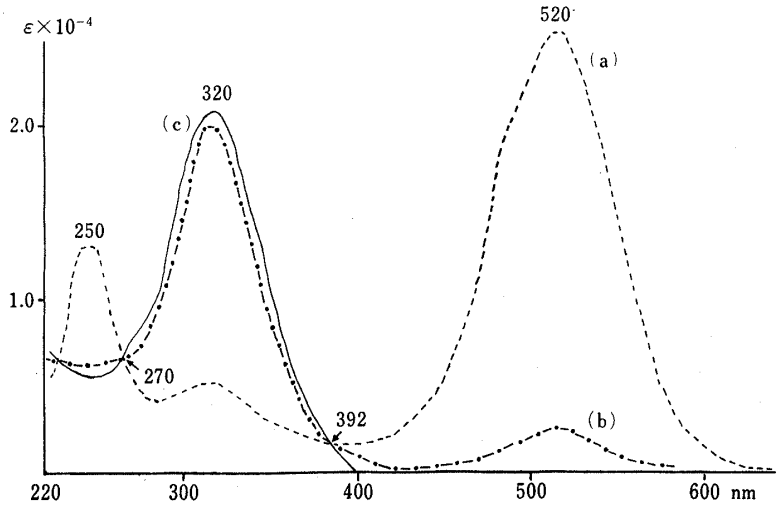

Fig. 2. Absorption Spectral Changes of **3** Neutralized with an Equivalent Amount of NH_3 in $CHCl_3$ after Standing for 60 min in Acidic Chloroform

(a) after standing for 60 min in acidic chloroform; (b) 3 min after being neutralized; (c) 10 min after being neutralized.

TABLE I. ^{13}C-NMR Chemical Shifts of **1**, **2**, **3**, **3A**, **3B**, DAB[a] and DAB (in HCl)

Carbon No.	**1**	**2**	**3**	**3A**	**3B**
1	37.0	37.2	37.2	37.1	37.1
2	27.8	28.0	28.0	28.0	28.0
3	73.8	80.3	80.2	80.2	80.2
4	38.1	38.7	38.7	38.7	38.7
5	139.7	140.9	140.8	140.8	140.8
6	122.3	121.3	121.3	121.2	121.2
7	32.1	32.3	32.3	32.2[c]	32.2[c]
8	31.2	31.3	31.3	31.1[c]	31.1[c]
9	50.0	50.2	50.2	50.1	50.1
10	36.7	37.0	37.0	37.0	37.0
11	20.9	21.0	21.0	20.9	20.9
12	39.5	39.6	39.6	39.5	39.5
13	43.3	43.3	44.0	44.0	44.0
14	55.0	55.1	55.2	55.2	55.2
15	34.1	34.2	34.3	34.2	34.2
16	84.3	84.2	84.0	84.2	85.1
17	64.2	64.3	65.2	65.3	64.3
18	13.9	14.0	14.1	14.1	14.6
19	19.3	19.4	19.4	19.4	19.4
20	103.7	103.3	106.1	108.3	108.7
21	11.6	11.6	13.2	13.1	12.3
22	151.6	151.9	153.3	151.6	149.1
23	23.2	23.4	129.5	129.2	41.4
24	30.8	31.1	32.6	32.4[c]	31.2[c]
25	32.1	32.9	32.3	32.2[c]	31.9[c]
26	69.1	78.3	78.1	77.7	77.7
27	16.7	16.9	17.2	17.0	17.0
$\underline{C}H_3CO$	21.3, 21.0				
$CH_3\underline{C}O$	170.3, 171.0				
CH_3O		55.5, 58.7	55.5, 58.6	55.5, 58.7	55.5, 58.7
	DAB	DAB (in HCl)			
1′	125.2	137.5	125.6	139.1,	139.5 [b]
2′, 6′	131.8	132.0	130.0	130.3,	130.8
3′, 5′	111.0	122.3	112.0	120.3,	120.4
4′	154.3	146.8	149.2	141.1,	141.4
CHO or C=$\underline{C}$H	189.9	190.3	131.8	134.5,	134.9
$(CH_3)_2N$	39.8	47.0	40.4	46.5	46.5

a) DAB: *p*-dimethylaminobenzaldehyde.
b) The assignments to **3A** or **3B** are unknown.
c) Assignments may be reversed.

by ^{13}C-NMR spectroscopy. The chemical shifts of steroidal C_1–C_{15} and C_{19} carbons are almost the same as those of **3**, but slight differences were observed in the signals of other carbons, C_{16}–C_{27}. On the other hand, the conspicuous changes in the chemical shifts of the aromatic ring carbons may be attributable to the conversion of an amine group on the aromatic ring to a quaternary amine. Moreover, each carbon of C_{16}–C_{25} and the benzylidenyl moiety except C_{23} shows a pair of signals having equal intensities in the ^{13}C-NMR spectrum of **3** in acidic deuterochloroform, and the signals at δ 129.2 (s) and 41.4 (d) ppm may be assigned to the C_{23} carbons of two tautomers.

Based on the UV and ^{13}C-NMR spectra, it was suggested that compound **3** exists in two tautomeric forms, **3A** and **3B**. The existence of the postulated tautomer (**3C**) has not been

proved and the possibility of the migration of the double bond from C_{20} to C_{22} is inconsistent with the conclusion of Hirschmann *et al.*[8] that the double bond migrates from C_{22} to C_{20} under acidic conditions.

Although the isolation of the colored substance from the Ehrlich reaction mixture of **2** has not been achieved, it was elucidated that the active site of **2** in this reaction is the C_{23} methylene group and that the coloration of the condensation product, **3**, in the acidic medium is due to the formation of the iminium cation (**3B**).

Further studies on the coloration mechanism of Ehrlich reagent with 22-hydroxy (or alkoxy)-furostanol saponins will be reported in a forthcoming paper.

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus (hot stage type) and are uncorrected. The IR spectra were recorded with a Shimadzu IR-27G spectrometer, UV spectra with a Shimadzu UV-200S spectrophotometer, ^{1}H-NMR spectra with a Varian CFT-20 spectrometer at 80 MHz and ^{13}C-NMR spectra with the same apparatus at 20 MHz. Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; dd, double doublet; m, multiplet; br, broad). The carbon signals of ^{13}C-NMR were assigned on the basis of the spectral data of diosgenin[9] and the substituent effect on the benzene ring.[10] Off-resonance spectra of each compound were also taken in order to aid assignment. Thin-layer chromatography (TLC) was performed on precoated silica gel plates and detection was achieved by spraying 10% H_2SO_4 followed by heating, or under irradiation with a UV lamp. Column chromatography was carried out on silica gel (Merck), Avicel (Funakoshi Co.) and Sephadex LH-20 (Pharmacia Co.).

Preparation of Ehrlich Reagent——Solution A: 1 g of *p*-dimethylaminobenzaldehyde (Wako Pure Chem. Ind.) in 100 ml of ethanol. Solution B: 30% hydrogen chloride in ethanol. Solutions A (200 ml) and B (50 ml) were mixed just before use.

Preparation of ψ-Diosgenin——According to Marker's method, diosgenin acetate (10 g, colorless needles from MeOH, mp 208—209 °C, $[\alpha]_D^{30}$ −134.3° (c=1.05, $CHCl_3$)), obtained by acetylation of diosgenin with acetic anhydride and pyridine, was treated with acetic anhydride (40 ml) in a sealed tube at 170 °C for 36 h. The reaction mixture was evaporated under reduced pressure and the residue was chromatographed on silica gel with hexane–AcOEt (10 : 1, v/v) to give diosgenin acetate (1.5 g) and ψ-diosgenin diacetate (**1**, 6.3 g), which was recrystallized from ethanol to afford colorless needles, mp 105 °C (dec.), (lit. 101—102 °C), $[\alpha]_D^{30}$ −44.2° (c=0.86, $CHCl_3$). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{31}H_{46}O_5$: C, 74.66; H, 9.30. Found: C, 74.39; H, 9.10.

ψ-Diosgenin diacetate (6 g) was refluxed with 5% KOH–MeOH for 10 min and the reaction mixture was post-treated to afford ψ-diosgenin (5.3 g) as colorless needles from Et_2O, mp 190—191 °C (dec.), (lit. mp 190—192 °C).

Methylation of ψ-Diosgenin——ψ-Diosgenin (5.0 g) was methylated by Hakomori's method.[11] After addition of water, the reaction mixture was extracted with ether and the extract was washed with water, dried and evaporated. The residue was purified by column chromatography on silica gel with hexane–AcOEt (10 : 1, v/v) and the product was recrystallized from methanol to give ψ-diosgenin dimethyl ether (**2**) as colorless needles (4.5 g), mp 105 °C (dec.), $[\alpha]_D^{30}$ −38.6° (c=1.0, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1695 (olefin). ^{1}H-NMR (in $CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.91 (3H, d, J=7 Hz, 27-CH_3), 1.01 (3H, s, 19-CH_3), 1.58 (3H, s, 21-CH_3), 3.31, 3.35 (each 3H, s, CH_3O), 4.70 (1H, m, C_{16}–H), 5.33 (1H, br, C_6–H). UV λ_{max}^{EtOH} nm (ε): 205 (10200), 220 (sh, 6600). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{29}H_{46}O_3$: C, 78.68; H, 10.47. Found: C, 78.44; H, 10.33.

Ehrlich Reaction of ψ-Diosgenin Dimethyl Ether (2)——A mixture of ψ-diosgenin dimethyl ether (**2**, 5 g) and Ehrlich reagent (solution A (200 ml) and solution B (50 ml)) was warmed at 40 °C for 30 min and the reaction mixture was kept in the dark for one week, then concentrated *in vacuo* at a low temperature. To the concentrate was added 200 ml of acetone. The acetone-insoluble material was collected by filtration and repeatedly recrystallized from methanol to give colorless needles, **3** (423 mg), mp 181 °C (dec.), $[\alpha]_D^{30}$ −86.3° (c=1.4, $CHCl_3$). UV $\lambda_{max}^{CHCl_3}$ nm (ε): 320 (20900); λ_{max}^{EtOH} nm (ε): 320 (26000), 207 (17000). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{38}H_{55}NO_3 \cdot 1/4H_2O$: C, 78.91; H, 9.67. Found: C, 78.87; H, 9.56.

The acetone-soluble portion was evaporated under reduced pressure. The residue (4.0 g) was subjected to column chromatography on Avicel with hexane–AcOEt (2 : 1, v/v, Fr. 1), followed by $CHCl_3$–MeOH–H_2O (10 : 1 : 0.1, v/v, the lower phase) to yield three fractions (Fr. II, III and IV). From Fr. II the starting material (**2**, 485 mg) and *p*-dimethylaminobenzaldehyde were recovered. The product obtained from Fr. III was recrystallized from methanol to give **3** (315 mg) as colorless needles. Fr. IV was concentrated to dryness and the red residue (1.35 g) was purified by column chromatography on Avicel with hexane–AcOEt (2 : 1, v/v), followed with $CHCl_3$–MeOH–H_2O (10 : 1 : 0.1, v/v, the lower phase). The amount of red substance in Fr. IV gradually decreased with each chromatography and, even though compound **3** had not been detected in Fr. IV, compound **3** was recovered in place of the red substance.

Properties of 3——The chloroform solution of **3** in methanol or chloroform becomes red upon addition of

hydrochloric acid or after the introduction of hydrogen chloride. The colored solution was examined by TLC (solvent: $CHCl_3$–MeOH–H_2O (8:2:0.2, v/v) and showed one red spot at *Rf* 0.42. The original compound **3** was recovered from the red solution after neutralization with solid Na_2CO_3, concentration, and extraction with $CHCl_3$. The recovered compound was identified as the original compound **3** by mixed fusion and by comparisons of IR and UV spectra.

UV Spectra of 3 in Acidic or Basic Solution——a) In Acidic Solution: The chloroform solution of **3** (0.018 mg/ml, 0.5 ml) was mixed with 3% hydrogen chloride solution prepared by dilution of 20% HCl–EtOH with $CHCl_3$ to make 10 ml. The absorption spectra of the solution were recorded over a period of 1 h after addition of hydrochloric acid. The results are illustrated in Fig. 1. b) Neutralization of Acidic Solution: The mixture of chloroform solution of **3** (0.016 mg/ml, 0.5 ml) and 3% HCl–$CHCl_3$ (4.75 ml) was kept for 60 min. An equivalent amount of 3% NH_3–$CHCl_3$ solution prepared by the introduction of dry ammonia gas into chloroform was added little by little, and UV spectra were recorded during the course of neturalization after removal of the NH_4Cl precipitate by filtration. The results are shown in Fig. 2.

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References and Notes

1) This work was presented at the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983.
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