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Michiya Kimura and Toshihiro Nishina : Fundamental Studies on Clinical Chemistry. XII.*1 On Some Aspects in the Colorimetries of Steroidal Compounds using *p*-Nitrophenylhydrazine. (1). Hydrazones derived from Δ^5-3-Hydroxysteroids.

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In the previous papers,[1~4] the present authors reported the colorimetric methods for micro amounts of Δ^5-3-hydroxysteroids such as cholesterol and dehydroepiandrosterone. The hydroxysteroids in acetone were oxidized to the corresponding Δ^4-3,6-dione derivatives *via* Δ^5-3-ketosteroids, which reacted subsequently with the *p*-nitrophenylhydrazine (PNPH)-hydrochloric acid reagent to form hydrazones; reddish purple colors showing the maximum light absorption at about 590 mμ were then produced from these hydrazones in alkaline dimethylformamide (DMF) solutions. The hydrazone thus obtained from cholesterol, which was employed as a representative of Δ^5-3-hydroxysteroids in this paper, showed slightly different light absorption from that of the authentic mono-, bis-*p*-nitrophenylhydrazone of cholest-4-ene-3,6-dione or of their mixtures and this could be observed when the reaction was carried out in such a highly diluted solution as about 7.5×10^{-5} mole/L. of the ketone.[1] The present paper deals with the enolation of the 3,6-dione and the hydrazones formed during the colorimetric procedures described above.

In general, the steroidal 3-keto group is more active than the corresponding 6-one.[5] When these ketones were subjected to the reaction with PNPH reagent under the direction in the above-mentioned colorimetric methods, the smaller light absorption of 3- and 6-keto derivatives has been shown as summarized in Table I.[6] Contrary to the 3-keto group,[1] the difficulty of the formation of hydrazones was thus

Table I.

	λ_{max} mμ	ε' [6]	Index[6]
Cholestan-3,6-dione	520	2100	7
Cholest-4-ene-3,6-dione	590	37900	124
Cholestan-3-one	520	6000	20
Cholestan-3β-ol-6-one acetate	520	2900	9
Cholest-4-en-6-one	540	2500	8
Cholest-4-en-3-one	540	30500	100
Cholest-4,6-dien-3-one	570	44600	146

*1 Part XI : This Bulletin, **15**, 454 (1967).

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1) M. Kimura, T. Nishina : Yakugaku Zasshi, **84**, 390 (1964).
2) *Idem* : This Bulletin, **12**, 521 (1964).
3) M. Kimura, I. Hariya, T. Nishina : Bunseki Kagaku, **14**, 125 (1965).
4) *Idem* : This Bulletin, **13**, 414 (1965).
5) L. F. Fieser : J. Am. Chem. Soc., **75**, 4377, 4386, 4395 (1953).
6) M. Kimura, T. Sakamoto, T. Nishina : This Bulletin, **15**, 454 (1967).

indicated on the 6-keto group, as has been observed in the case of cholest-4-ene-3,6-dione mono-2,4-dinitrophenylhydrazone.[5] Since cholestan-3,6-dione gave weaker coloration than cholestan-3-one as also shown in the same Table, the vicinal 6-keto group might affect on the formation of hydrazone, that has been discussed by Djerassi, *et al.*[7] from the point of view of the optical rotatory dispersion on this dione.

When the methanolic or ethanolic solution of cholest-4-ene-3,6-dione (I) was heated with hydrochloric acid at 60° for 60 min., a new maximum of light absorption which was stable also after neutralization with alkali, appeared at 306 mμ together with the initial one at 252 mμ. The alcoholic solution thus obtained reacted with the PNPH reagent to give a maximum absorption at 585 mμ, identical with that derived directly from I using the same reagent. The intensity of the absorption at 306 mμ attained a maximum soon after 3 min. when I was heated in such acidic solution. On the contrary, the formation of hydrazone from I by the PNPH reagent containing sufficient amounts of hydrochloric acid was observed to be equilibrated late after about 40 min. as shown in Fig. 1. Cholest-4-ene-3,6-dione 6-enol ethyl ether (II) has well been known to be formed from I under refluxing in acidic absolute ethanol,[8~10] which was obtained also by the present authors giving m.p. 165~166° and maximum absorption at 306 mμ; although the reaction mixture in acidic absolute methanol gave the maximum at the same wave length, no corresponding methyl ether was isolated in a pure state. These results might suggest that in common with these colorimetric procedures, Δ^5-3-hydroxysteroids were first oxidized into the corresponding Δ^4-3,6-diones which reacted then with alcohol into their 6-enol ethers under the presence of hydrochloric acid and their mono-hydrazones were finally formed.

It may reasonably be expected from the point of view of analytical chemistry to introduce a novel method for the detection of Δ^5-3-hydroxysteroids by use of this color reaction. The same steroids may more strictly be detected also by the light absorption at 252 mμ of the corresponding Δ^4-3,6-diones formed under such a controlled chromic acid oxidation or further by the two maxima at 252 and 306 mμ of their 6-enol ethers derived as mentioned above. From their olefinic isomers, Δ^4-3-hydroxysteroids, they may be differentiated by the light absorption of the derivatives formed under the same oxidation, that occurred at 240 mμ without any subsequent shift even after being refluxed in an acidic or alkaline solution.[11]

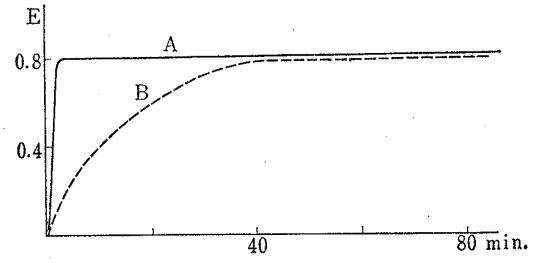

Fig. 1.

A: Treatment of Δ^4-3,6-dione (100 μg.) with MeOH-HCl and determined at 306 mμ in MeOH
B: Treatment of Δ^4-3,6-dione (50 μg.) with PNPH reagent and measured at 585 mμ in alkaline DMF

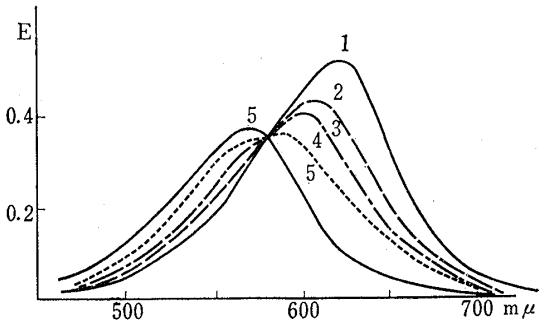

Fig. 2.

	III	IV
1:	2.0 ml.	+ 0.0 ml.
2:	1.5 ml.	+ 0.5 ml.
3:	1.0 ml.	+ 1.0 ml.
4:	0.5 ml.	+ 1.5 ml.
5:	0.0 ml.	+ 2.0 ml.

7) C. Djerassi, W. Closson: J. Am. Chem. Soc., **78**, 3761 (1956).
8) A. Windaus: Ber., **39**, 2249 (1906); **40**, 257 (1907).
9) W. C. J. Ross: J. Chem. Soc., **1946**, 737.
10) A. Butenandt, G. Schramm: Ber., **69**, 2289 (1936).
11) L. L. Smith: Steroids, **1**, 544, 570 (1963).

When II came into reaction with PNPH under usual condition, mono-hydrazones, m.p. 272~273°(III) as well as m.p. 286~288°(IV), and cholest-4-ene-3,6-dione bis *p*-nitrophenylhydrazone, m.p. 206~208°(V), were derived in an approximate ratio of 50:1:10. Infrared spectrum of III revealed unexpectedly the carbonyl absorption at about 1650 cm^{-1} and the N-H stretching vibration at 3280 cm^{-1} in KBr as well as Nujol and at 3360 cm^{-1} in chloroform. In the case of IV, on the contrary, no carbonyl vibration but the absorption at 3355 cm^{-1} in Nujol and chloroform was observed, revealing that there was no difference between its states of solid and liquid. Cholest-4-ene-3,6-dione mono-*p*-nitrophenylhydrazone which was prepared as a reference substance from I in diethyleneglycol diethyl ether so that the formation of 6-enol ether may be excluded, was quite identical with III. Therefore, IV would reasonably be regarded as cholest-4-ene-3,6-dione 6-enol ethyl ether *p*-nitrophenylhydrazone.

The colorimetric procedure on Δ^5-3-hydroxysteroid in highly reduced amount gave the absorption curve having maximum with shoulder at about 590 and 570 mμ, respectively,[4] which was far different from that of V and slightly from those of III as well as IV but was quite similar to that of the mixture of these mono-hydrazones. The absorption spectra of these mixtures prepared under the different ratios in concentration were measured as shown in Fig. 2, indicating that the hydrazones, III and IV, may be formed in an approximate ratio of 1:1 in the afore-mentioned colorimetric procedures for micro quantities of Δ^5-3-hydroxysteroids.

Experimental

Preparation of Reference Substances

Cholest-4-ene-3,6-dione 6-Enol Ethyl Ether (II)——Derived from I by the method of Windaus,[8] employing conc. H_2SO_4 and EtOH. Purified through silica gel chromatography and recrystallized from MeOH–ether m.p. 165~166°.

Cholest-4-ene-3,6-dione Mono *p*-Nitrophenylhydrazone (III)——A mixture of 0.5 g. of I, 0.2 g. of PNPH and 1.0 ml. of conc. HCl in 10 ml. of diethyleneglycol dimethyl ether was heated at 70° for 10 min. Evaporation of the filtrate under reduced pressure left residue which was recrystallized to yellow crystals, m.p. 273~274°(decomp.)*3 from $CHCl_3$–EtOH. No significant depression was observed on the mixed melting point with that isolated from the reaction mixture as described below. *Anal.* Calcd. for $C_{33}H_{47}O_3N_3$: C, 74.26; H, 8.88; N, 7.87. Found: C, 74.07; H, 8.69; N, 8.02. IR ν_{max}^{KBr} cm^{-1}: 3280 (N-H), 1645 (C=O), 1605 (C=C). $\nu_{max}^{CHCl_3}$ cm^{-1}: 3360, 1670, 1600. UV $\lambda_{max}^{CHCl_3}$ mμ: 411. λ_{max}^{DMF} mμ (ε): 428 (5.16×10^4). $\lambda_{max}^{DMF\text{-}NaOH}$ mμ (ε): 612 (6.44×10^4).

Cholest-4-ene-3,6-dione Bis-*p*-Nitrophenylhydrazone (V)——Derived from I, as has been reported previously.[1]

Reaction of Cholest-4-ene-3,6-dione (I) with Hydrochloric Acid in Alcohol and with *p*-Nitrophenylhydrazine (PNPH) Reagent[4] (Fig. 1)——To 1.0 ml. of ethanolic (or methanolic) solution containing 100 μg. of I was added 1.0 ml. of HCl–EtOH solution which was prepared with 2.0 ml. of conc. HCl and 48.0 ml. of EtOH. The solutions thus formed were heated at 60° for different periods. The increase in absorbance at 306 mμ was observed as a function of time as shown on the curve A in Fig. 1. The solution heated with PNPH reagent at 60° for 60 min. gave the maximum absorption at 585 mμ, when 10.0 ml. of DMF and 0.5 ml. of 1*N* NaOH were then added.

Mixtures, each containing 0.5 ml. of PNPH reagent and 50 μg. of I in 0.5 ml. of MeOH, were heated at 60° for different periods. After cooling, 0.5 ml. of 1*N* NaOH and 5.0 ml. of DMF were added to each solution thus obtained. Color development at 585 mμ was observed as a function of time as shown on the curve B in Fig. 1.

Separation of *p*-Nitrophenylhydrazones——A mixture of 1.0 g. of II, 0.4 g. of PNPH and 1.0 ml. of conc. HCl in 80 ml. of EtOH was heated at 70° for 15 min. The precipitates formed were submitted to chromatography on silica gel (15 g.) employing $CHCl_3$ as an eluent.

III——Removal of solvent from the eluate left the residue that was recrystallized from $CHCl_3$ to give 1.0 g. of yellow plates, m.p. 272~273°(decomp.). IR $\nu_{max}^{KBr\ (or\ Nujol)}$ cm^{-1}: 3280, 1650, 1605. $\nu_{max}^{CHCl_3}$ cm^{-1}: 3360,

*3 The hydrazone prepared from the methanolic reaction mixture gave the melting point of 259~261° (decomp.).[1]

1670, 1600. UV λ_{max}^{DMF} mμ (ε): 427 (5.2×10^4), $\lambda_{max}^{DMF\text{-}NaOH}$ mμ (ε): 618 (6.88×10^4). *Anal.* Calcd. for $C_{33}H_{47}O_3N_3$: C, 74.26; H, 8.88; N, 7.87. Found: C, 74.99; H, 8.84; N, 7.80.

V——Sufficient amount of water was added to the filtrate that was obtained from the reaction mixture mentioned above, giving precipitate which was submitted to chromatography on silica gel (15 g.). The eluates with $CHCl_3$ were divided into two fractions according to the adsorption bands on the column. Removal of solvent from the first fraction left the residue that was recrystallized from $CHCl_3$ to give 0.2 g. of red needles, m.p. 206~208°(decomp.). IR ν_{max}^{Nujol} cm^{-1}: 3300, 1600. UV $\lambda_{max}^{DMF\text{-}NaOH}$ mμ (ε): 535 (8.0×10^4), 670 (4.4×10^4).

Cholest-4-ene-3,6-dione 6-Enol Ethyl Ether *p*-Nitrophenylhydrazone (IV)——The second fraction gave 20 mg. of orange needles, recrystallized from $CHCl_3$, m.p. 286~288°(decomp.). IR $\nu_{max}^{Nujol\ (or\ CHCl_3)}$ cm^{-1}: 3355, 1600. UV λ_{max}^{DMF} mμ (ε): 423(3.17×10^4), $\lambda_{max}^{DMF\text{-}NaOH}$ mμ (ε): 558 (4.11×10^4). *Anal.* Calcd. for $C_{35}H_{51}O_3N_3$: C, 74.82; H, 9.15; N, 7.48. Found: C, 74.68; H, 8.67; N, 7.12.

Absorption Spectra on Mixtures of III and IV (Fig. 2)——To ethanolic solutions containing III (1.390 mg./dl.) and IV (1.422 mg./dl.), respectively, were prepared as the working standard solutions which were then combined into test tubes so that different ratios in concentration of the hydrazones were formed. After removal of solvent from each tube, 5.0 ml. of DMF and 0.5 ml. of 1*N* NaOH were added to observe the spectrum.

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In the previous papers[1~4] of this series, the colorimetric methods for some steroidal compounds using *p*-nitrophenylhydrazine (PNPH) reagent were reported. One of the advantages of these methods is the favorable indifference of PNPH reagent which would otherwise remain in excess amounts so that highly interfering reagent blank should be anticipated in ordinary solvents. Nakamura and Yoshida[5] reported the decomposition of PNPH to *p*-nitrophenol and molecular nitrogen in alkaline dimethylformamide (DMF) solution, giving maximum absorption at 420 mμ. The authors have also detected the phenol on thin-layer chromatogram,*3 which was colorimetrically estimated as an unexpectedly smaller quantities as is discussed below. The present paper deals with the decomposition of PNPH in alkaline solutions that was studied spectroscopically as well as gas-chromatographically.

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*1 Part XII: This Bulletin, **15**, 1239 (1967).

*2 Nishi-6-chome, Kita-12-jo, Sapporo (木村道也, 仁科甫啓).

*3 Rf. 0.40 on Wako-Gel B-5 using solvent system of $CHCl_3$–benzene–MeOH (10:10:1).

1) M. Kimura, Y. Sakai, T. Nishina: Steroids, **4**, 255 (1964).
2) M. Kimura, T. Nishina: This Bulletin, **12**, 521 (1964).
3) *Idem*: *Ibid.*, **15**, 454 (1967).
4) M. Kimura, I. Hariya, T. Nishina: *Ibid.*, **13**, 414 (1965).
5) N. Nakamura, T. Yoshida: Bunseki Kagaku, **11**, 669 (1962).