1670, 1600. UVλ_{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. UVλ_{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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Michiya Kimura and Toshihiro Nishina: Fundamental Studies on Clinical Chemistry. XIII.[*1] On Some Aspects in the Colorimetries of Steroidal Compounds using *p*-Nitrophenylhydrazine. (2). Decomposition of *p*-Nitrophenylhydrazine in Alkaline Dimethylformamide Solution.

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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.

[*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).

Experimental

Reagents and Apparatus——*p*-Nitrotoluene standard solution : In 100 ml. of dried benzene, 200 mg. of *p*-nitrotoluene, m.p. 53~54°(from MeOH), was dissolved.

Nitrobenzene standard solution : In 100 ml. of dried benzene, 200 mg. of nitrobenzene (Reagent Grade) was dissolved.

Sodium hydroxide solution : Aqueous solution was prepared from conc. NaOH solution, removed precipitate of carbonate by filtration through a glass filter.

All other reagents and solvents were used with further purification.

Spectrophotometer : Absorption spectra and absorbances were measured using Hitachi Model EPS-2U Recording as well as RSP-2 Rapid Scanning Spectrophotometers and Hitachi Model EPU-2A Photo-electric Spectrometer, respectively.

Gas Chromatograph : Identifications of nitrogen and determination of nitrobenzene were performed using Yanagimoto Model GCG-2 and Shimadzu Model GC-1B-HFD Gas Chromatographs, respectively.

Absorption Spectra (Fig. 1)——A : Two solutions, one containing 100 μg. of PNPH and the other containing 0.2 ml. of *N*/100 NaOH in each 5 ml. of DMF kept in different tubes were blended instantaneously in a mixing cell to be observed on a rapid scanning spectrophotometer without delay.

B : A mixture of 27.5 μg. of PNPH (m.p. 157~158°) and 0.2 ml. of 1*N* NaOH in 4 ml. of MeOH was measured.

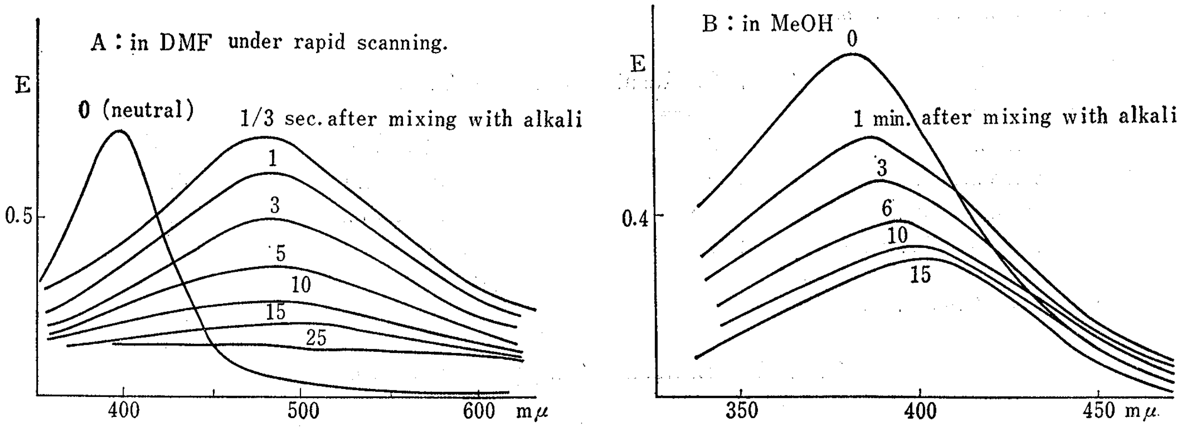

Fig. 1. Absorption Spectra shown during the Course of Decomposition of PNPH in Alkaline Solutions

Identification of Molecular Nitrogen (Fig. 2)——Molecular nitrogen produced in a gas cylinder from the reaction mixture of *ca.* 100 mg. of PNPH and 2 ml. of 2*N* NaOH in 5 ml. of DMF was submitted to gas chromatography over Molecular-sieve 5A (60~80 mesh); He was employed as carrier gas (80 ml./min.) and column temperature was kept at 30°.

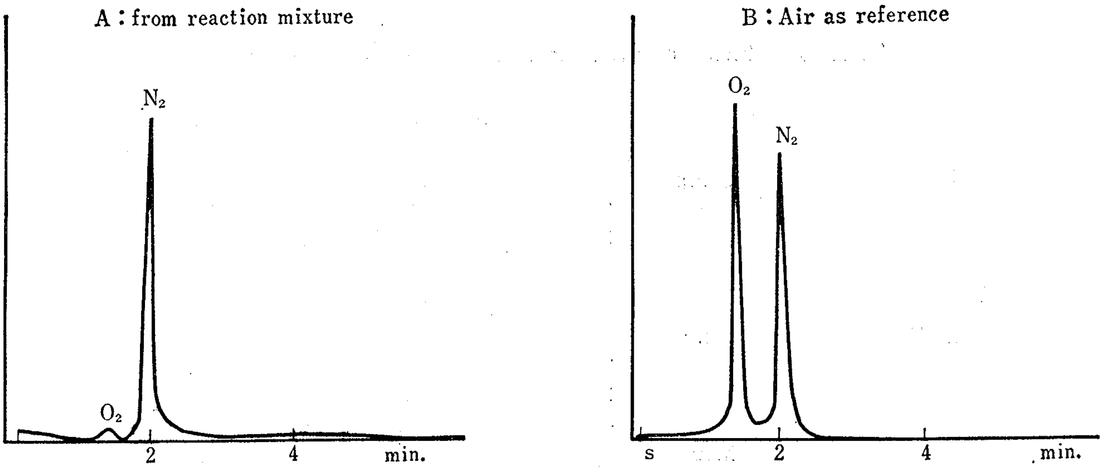

Fig. 2. Gas Chromatographic Tracing of Molecular Nitrogen generated from *p*-Nitrophenylhydrazine in Alkaline Dimethylformamide

Column condition : Molecular-sieve 5A (60~80 mesh);
He : 80ml./min.; Col. temp. : 30°.

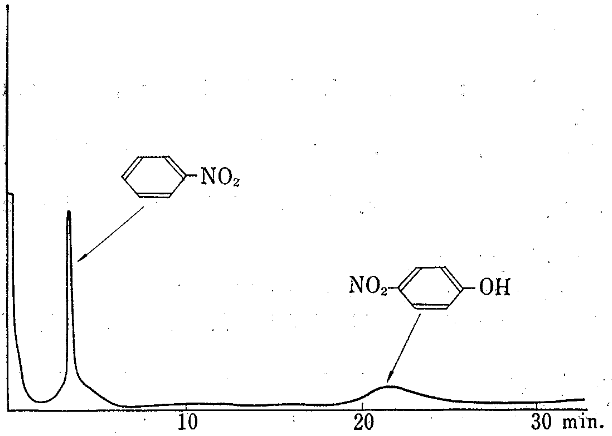

Fig. 3. Gas Chromatogram of Decomposition Products of PNPH in Alkaline DMF

Column condition: 1.5 m.×4 mm. i.d. column; 30% Siliconegrease on Celite 545 (80~100 mesh.); Col. Temp.: 140°; N_2: 40 ml./min. (2 kg./cm^2). *p*-Nitrotoluene time: 7.0 min.

Determination of Nitrobenzene——A solution of 6.09 mg. of PNPH in 10 ml. of DMF was made alkaline with 1 ml. of 2.5*N* NaOH and was kept at room temperature for 60 min. To 0.25 ml. of the reaction mixture, 3 ml. of *p*-nitrotoluene standard solution was added after neutralized with 2*N* HCl and the solution thus obtained was extracted with 30 ml. of benzene, a quite suitable solvent as shown in Table II. After being shaken out three times with each 10 ml. of saturated aq. NaCl, dried over 10 g. of anhydrous Na_2SO_4 overnight,[*4] filtered off and finally evaporated to dryness under reduced pressure, the benzene layer left the residue which was then dissolved in 0.2 ml. of Me_2CO and submitted to gas chromatography as shown in Fig. 3.

Nitrobenzene produced was estimated from the calibration diagram (peak height ratio to weight ratio of nitrobenzene/nitrotoluene) as summarized in Table III.

Table I. Relative Retention Times

Aniline	0.32	*p*-Nitroanisol	2.10
Nitrobenzene	0.54	*p*-Nitrophenol	4.33
p-Nitrotoluene	1.00[a]	*p*-Nitroaniline	5.46
m-Dinitrobenzene	2.48		

a) Time, 7.0 min.

Table II. Extraction Test for Nitrobenzene

		Ether (%)	Benzene (%)
Sample	1	82.2	93.8
	2	82.1	100.0
	3	79.9	103.8
	4	75.5	—
Mean		79.8	99.2

Table III. Yields of Products in Decomposition of PNPH

			Nitrobenzene (%)	*p*-Nitrophenol (%)[a]
DMF	Sample	1	98.8	< 1
		2	88.8	
		3	98.8	
		4	98.8	
	Mean		96.3	
MeOH	Sample	1	71.4	20.1
		2	77.7	19.8
		3	74.2	19.8
		4	64.4	18.7
	Mean		71.9	19.6

a) Determined colorimetrically at 427 mμ in DMF.

[*4] That was found to be quite important to avoid the larger dispersion of analytical data.

Results and Discussion

When alkali was added to the PNPH solutions, the color[6] once produced immediately faded away so fast as in several seconds of half-life and the measurement of the absorption spectrum was practically impossible by the ordinary spectrophotometer. No maximum absorption at 394 mμ appeared in neutral DMF solution of PNPH was observed on the rapid scanning spectrophotometer even at the elapsed time of 0.35 seconds immediately after adding alkali to the solution as shown in Fig. 1 (A). In an alkaline methanolic solution, on the contrary, no maximum at about 480 mμ observed in the alkaline DMF was recognized on the same spectrophotometer and rather slowly the maximum at about 400 mμ due to *p*-nitrophenol appeared instead as shown in the same Figure (B), which was estimated colorimetrically as about 20% in the yield as shown in Table III. The fading, however, was highly reduced down to take several minutes, when the reaction was carried out strictly under nitrogen atmosphere in DMF.

The gaseous substance generated at the decomposition of PNPH in an alkaline DMF was identified as molecular nitrogen gas-chromatographically as shown in Fig. 2. No molecular hydrogen was detected even at the higher sensitivity of the detector. Gas chromatography of the other products indicated the presence of nitrobenzene and trace amount of *p*-nitrophenol as shown in Fig. 3; the former was determined quantitatively by the same chromatography using *p*-nitrotoluene as an internal standard that is summarized in Table III.

The results obtained in this study indicated that PNPH undergoes oxidative decomposition in an alkaline solution, forming nitrobenzene as well as molecular nitrogen as the main products and far smaller quantities of *p*-nitrophenol. The alkaline solution of 2,4-dinitrophenylhydrazine produced *m,m'*-dinitroazoxybenzene, 1-hydroxy-6-nitro-1,2,3-benztriazole and *m*-dinitrobenzene, which were recognized to be variably formed depending on the differences of alkali and solvent employed.[7] The decomposition of PNPH still proceeded even under the nitrogen atmosphere indicated the presence of other hydrogen acceptor than the dissolved oxygen and nitro group in PNPH as well as nitrobenzene formed might thus be considered as the possible ones. However, molecular oxygen dissolved in DMF may be the principal oxidant in such a diluted solution as in the cases of the colorimetry in question. Cram and Bradshaw[8] studied on the oxidative and base-catalyzed decomposition of alkylhydrazines to alkylhydrides and molecular nitrogen *via* alkyldiimides. Aromatic derivatives of hydrazine have also been known to proceed in a similar way.[9,10] Therefore, after the addition of alkali the acidic imino-hydrogen of PNPH in DMF may first be eliminated to give the anion II showing the maximum absorption at about 480 mμ,[*5] which may in turn be easily oxidized to nitrobenzene and molecular nitrogen *via* labile diimide anion III by the dissolved oxygen and the base-catalysis as shown in Chart 1.

Alkaline hydrolysis of *p*-nitroaniline has been well known to give *p*-nitrophenol quite easily. In methanol as a protic solvent, the acidity of imino-hydrogen in PNPH would become lower than in DMF as a dipolar aprotic solvent[11] and the activity of hydroxide anion would also be in a similar situation that will be discussed in the following paper.

[*5] In alkaline DMF *p*-nitroaniline and its N-methyl derivative gave the maximum absorption at 467 and 485 mμ, respectively (unpublished).

6) J. Moir : J. Chem. Soc., **1924**, 1549.

7) A.K. Macbeth, J.R. Price : *Ibid.*, **1934**, 1637; **1937**, 982.

8) D.J. Cram, J.S. Bradshaw : J. Am. Chem. Soc., **85**, 1108 (1963).

9) F.D. Chattaway : J. Chem. Soc., **1907**, 1323, 1326; **1908**, 270; **1909**, 1065.

10) E.H. Rodd : "Chemistry of Carbon Compounds" Vol. III-A, p. 345.

11) A.J. Parker : Quart. Rev., **16**, 163 (1962).

As is shown in Fig. 1, PNPH in an alkaline methanol seemed to be hardly dissociated and thus to obtain more probabilities of the formation of intermediate anion I than in DMF so that the additional pathways may subsequently become available as shown in Chart 1.

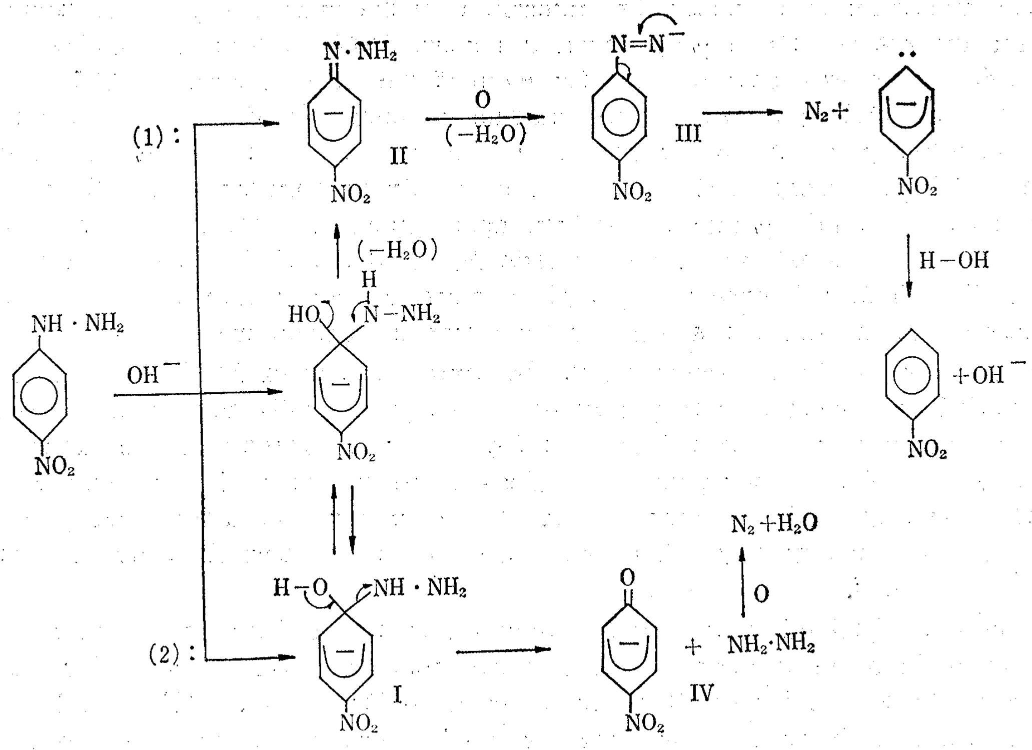

Chart 1.

(1) : mainly proceeded in the solutions of both dimethylformamide and methanol.
(2) : accompanied in the case of methanolic solution.

In the practice of the colorimetry using *p*-nitrophenylhydrazine presented by the authors,[1~4)] it was directed to add alkali to the methanolic reaction mixture before making DMF solution. The above-mentioned results, however, would recommend that DMF should be added beforehand in order to avoid the formation of *p*-nitrophenol so that the reagent blank may more favorably be reduced down.

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