**Biochemical Studies on Glucuronides. IV.¹⁾ Cleavage
of *p*-Nitrophenyl Glucuronide by Ultraviolet
Light Irradiation at Alkaline Medium**

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Ultraviolet light irradiation was applied to the study of nonenzymatic dissociation of *p*-nitrophenyl glucuronide (PNG) at low temperature in acidic, neutral and alkaline conditions. The cleavage of PNG was negligible at acidic or neutral medium, but it was almost quantitative and the production of *p*-nitrophenol (PN) was over 90% at alkaline condition. Nevertheless, the recovery of sugar moiety of PNG was very low, and glucuronic and glucaric acids were formed only 5–8%, and under 1%, respectively.

The study on the nonenzymatic dissociation of O-glucuronides of *p*-nitrophenol (PNG), and of phenolphthalein (PMG) has been reported from this laboratory,³⁾ and these glucuronides have been found that they are split into aglycones and carbohydrate moieties in solutions with L-ascorbic acid or hydrogen peroxide or hydrogen peroxide plus metal ion system. Inorganic metal ions, such as cupric and ferrous ions were effective to promote the dissociation reaction catalytically.

In the previous paper,⁴⁾ the authors reported the formation of glucuronic acid and *p*-nitrophenol as the primary dissociation products of PNG, however, besides these substances, glucaric acid and 4-nitrocatechol, which were the oxidation products of their parent compounds, were also observed. Therefore, the presence of glucaric acid and 4-nitrocatechol suggested that oxidation would be involved in the reaction along with dissociation process, and the formation and the contribution of hydroxyl radical·OH could be considered.

Many studies have supported the effectiveness of ultraviolet light irradiation (UV irradiation) to promote the formation of free radicals in organic compounds excited with its high energy.⁵⁾

To elucidate the reaction mechanism of dissociation, UV irradiation was applied to PNG-hydrogen peroxide-Fe²⁺ system at acidic condition,¹⁾ and the acceleration of the cleavage of PNG was clearly observed.

Considering from the above result, UV irradiation seemed definitely to have some effect on the promotion of cleavage reaction of PNG.

Therefore, the cleavage effect of UV irradiation without any metal ions or other radical-producing reagents was further investigated, and this paper reports the UV irradiation study in detail.

1) This paper was read at the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1968. Part III: Y. Yamane, M. Miyazaki, K. Sakai and C. Ajiki, *Yakugaku Zasshi*, **89**, 863 (1969).

2) Location: Yayoi, Chiba.

3) Y. Yamane, K. Sakai and K. Ikeguchi, *Yakugaku Zasshi*, **87**, 227 (1967).

4) Y. Yamane, M. Miyazaki and K. Sakai, *ibid.*, **88**, 191 (1968).

5) For examples: R.A. Ferrari and F.P. Luduena, *Arch. Intern. Pharmacodyn.*, **156**, 405 (1965); J.H. Stocker and D.H. Kern, *J. Org. Chem.*, **33**, 291 (1968).

Results and Discussion

Effect of Reaction Medium

UV irradiation was carried on $10^{-3}M$ of PNG for 20 min at acidic, neutral, and alkaline conditions, respectively.

The adjusting of pH of the solution was made with 1 N HCl or 1 N NaOH.

Before UV irradiation study, the stability of PNG was checked, and PNG was found stable in the same mediums mentioned above.

In the control experiment, *p*-nitrophenol (PN) was found stable at any of these conditions. After the irradiation, the reaction mixture was brought to neutral, and further to about pH 10 using a borate buffer. The optical density of the solution was measured at $400 m\mu$ to determine the amount of PN which was liberated during the reaction. The results are shown in Tables I and II.

TABLE I. Effect of the Reaction Medium

Medium	Production ratio of PN (%)
Acidic 1N HCl	1
Neutral H ₂ O	2
Alkaline 1N NaOH	90

irradiation time: 20 min
sample solution: $0.979 \times 10^{-3}M$ PNG 0.10 ml + medium 0.90 ml
temperature: $2 \pm 1^\circ$

TABLE II. Stability of PN in Various Medium on UV Irradiation

Irradiation time Medium (N)		20 min (%)	60 min (%)
HCl	1.0	100	—
H ₂ O		100	—
NaOH	0.1	99	97
	1.0	98	96
	1.5	99	95
	2.0	99	95
	3.0	98	95

sample solution: $1.64 \times 10^{-3}M$ PN 0.1 ml + medium 0.90 ml
temperature: $2 \pm 1^\circ$

The production ratio of PN was very low, and only 1—2% at neutral or acidic condition, however, nearly 90% of PN was formed at alkaline condition. Therefore, the alkaline condition seemed favorable for the cleavage of PNG by UV irradiation, and various concentrations of NaOH solutions to be added to the sample solutions were examined to obtain the best basicity of the sample solutions.

Basicity of the Sample Solutions

NaOH concentrations and the irradiation time were examined for the cleavage of PNG. The figures in Table III mean the ratio of produced PN to the initial PNG. As is seen in Table III, the production of PN amounted to 30% at 0.1 N NaOH, while over 90% of PN was produced at 1 N NaOH under 20 min irradiation.

On more concentrated NaOH solutions than 1 N, the promoting effect to the cleavage was not observed. Consequently, about 1 N NaOH seemed the most favorable alkaline concentration.

TABLE III. Effect of Various NaOH Concentrations on the Cleavage of PNG

<div> <div> Irradiation time NaOH concent. (N) </div> </div>	20 min (%)	60 min (%)
0.1	30	45
1.0	90	87
1.5	91	92
2.0	91	89
3.0	91	90

sample solution: $0.979 \times 10^{-3}M$ PNG 0.10 ml + NaOH 0.90 ml
temperature: $2 \pm 1^\circ$

Under 60 min irradiation, 45% of PN was produced at 0.1 N NaOH, however, almost identical production ratio of PN was obtained at over 1 N.

This phenomenon suggests that the cleavage of PNG proceeds more rapidly at 1 N than at 0.1 N, however, the liberated PN may be susceptible for the subsequent decomposition at higher alkaline conditions during 60 min irradiation.

Stability of PN at the Condition of UV Irradiation for PNG

In order to understand the phenomenon observed on the irradiation, the stability of PN was investigated at the same conditions used for the cleavage of PNG.

Table II shows the decomposition of PN, which was 1–2% at the condition in the range of 0.1–3.0N NaOH under 20 min irradiation, while 4–5% at the same range of NaOH concentration for 60 min irradiation. Hence, it may be possible to determine the degree of cleavage of PNG by measuring the amount of PN under short time UV irradiation of PNG.

Direct Determination of Unchanged PNG

Moreover, the unchanged PNG was directly determined by measuring the increased amount of PN produced by acid hydrolysis of the unchanged PNG after the irradiation. As is seen in Table IV, the hydrolysis was made effectively on heating the PNG in 3 N HCl medium for 2 hr. PN was found stable at the same condition.

TABLE IV. Hydrolysis of PNG by 3N HCl

Heating time (min)	20	40	60	120	240
Hydrolysis ratio (%)	53	76	88	98	101

Hydrolysis ratio was estimated from the production ratio of *p*-nitrophenol.
sample solution: $0.979 \times 10^{-3}M$ PNG 0.10 ml + 3N HCl 3.0 ml

Cleavage of PNG on UV Irradiation

Fig. 1 shows the relation between the cleavage of PNG and the production of PN, and the production ratios of PN, glucuronic and glucaric acids are also given in Fig.2.

The difference between the cleavage ratio of PNG and the production ratio of PN was about 10% within 5–40 min irradiation, and this difference also increased at 60 min irradiation.

Therefore, about 10% of PNG was assumed to be converted to the unknown product besides PN before or on the cleavage reaction of PNG. The results observed at 60 min irradiation might be understood as the further decomposition of PN initially formed at the early stage of irradiation. As for the sugar moiety of PNG, only 5–8% of glucuronic acid was observed and glucaric acid was estimated only under 1%. Considering from the preliminary experiment that glucuronic acid was stable under UV irradiation at the same condition,

Fig. 1. Cleavage Ratio of PNG (a) and Production Ratio of PN (b) on UV Irradiation

sample solution: $1.00 \times 10^{-2}M$ PNG 0.10 ml
+ 2.0N NaOH 0.90 ml
temperature: $2 \pm 1^\circ$

Fig. 2. Production Ratios of *p*-Nitrophenol (a), Glucuronic Acid (b) and Glucaric Acid (c) on UV Irradiation of PNG

sample solution: $1.00 \times 10^{-2}M$ PNG 0.10 ml + 2.0N NaOH 0.90 ml
temperature: $2 \pm 1^\circ$

therefore, the fact that both of the amounts of glucuronic and glucaric acids were quite small, suggests the preceding chemical denaturation of glucuronic acid moiety of PNG before the cleavage or when cleavage occurs.

Experimental

Materials—*p*-Nitrophenyl- β -D-glucopyranosiduronic acid (PNG), and D-glucuronic acid were the preparations of Chugai Pharmaceutical Co. Sodium hydroxide was the analytical grade reagent of Wako Pure Chemical Industries. *p*-Nitrophenol (PN) was the preparation of Wako Pure Chemical Industries and recrystallized from benzene for our experiments. Glucaric acid was obtained from Tokyo Biochemical Research Institute.

UV Irradiation—UV irradiations were performed using a water-cooling type 100 W Ultraviolet Light Oscillator NY-2 (maximum intensity, 3650–4100 Å, Ohsawa Ultraviolet Light Co.). 0.1 ml of PNG ($1.00 \times 10^{-2}M$ or $0.979 \times 10^{-3}M$) was taken into a Petri dish (4.0 cm in diameter, and 1.6 cm in depth) and 0.9 ml of NaOH or HCl solution or water was added. The vessel was cooled in ice-water bath for a few min in dark, and then subjected to the irradiation. The irradiation distance was maintained at 5 cm from the center of the lamp to the bottom of the Petri dish.

Determination of *p*-Nitrophenol (PN)—In the case of the solution of low PNG concentration ($0.979 \times 10^{-3}M$), the irradiated sample was neutralized with H_2SO_4 or NaOH and transferred into a 25 ml graduated test tube with a glass stopper, and then 0.05M sodium borate–0.05 M sodium carbonate buffer solution (pH 10.0) was added to make the solution up to 10.0 ml. The absorbancy of the solution was determined at 400 m μ by a Bausch and Lomb Spectronic 20 colorimeter. As for the solution of high PNG concentration ($1.00 \times 10^{-2}M$), the irradiated sample was neutralized with H_2SO_4 and transferred into a 25 ml graduated test tube, and then diluted with water to make the solution up to 10.0 ml (solution A). 2.0 ml aliquot of solution A was taken into a 25 ml graduated test tube and borate buffer was added to make the volume to 10.0 ml. The absorbancy of this diluted solution was measured at 400 m μ .

Determination of Unchanged PNG after UV Irradiation—To determine the unchanged amount of PNG, acid hydrolysis of the PNG contained in the reaction mixture was performed. 2.0 ml aliquot of solution A was taken into a 25 ml test tube and 2.0 ml of 6 N HCl was added and the whole solution was heated in a boiling water bath for 2 hr. After cooling with water, the solution was neutralized with 2 N NaOH, and then made up to 20 ml using borate buffer (pH 10). The absorbancy was measured at 400 m μ for this solution, and the unchanged amount of PNG was obtained from the increment of PN before and after acid hydrolysis of PNG.

Determination of Glucuronic Acid—Determination of glucuronic acid was carried by the method of Sadahiro, *et al.*⁶⁾ The method was applied to 4.0 ml aliquot of solution A, and the total amount of glucuro-

6) R. Sadahiro, Y. Hinohara and A. Yamamoto, *J. Biochem.*, **57**, 815 (1965).

nic acid and glucuronic acid moiety in the unchanged PNG was determined together. The amount of glucuronic acid was then calculated by subtracting the amount of the unchanged PNG from the total amount.

Determination of Glucaric Acid—Glucaric acid in the irradiated sample was determined by the method of Ishidate, *et al.*⁷⁾

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7) M. Ishidate, M. Matsui and M. Okada, *Anal. Biochem.*, **11**, 176 (1965).