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202. Tadashi Okabayashi and Akihiro Yoshimoto : Reduction of 4-Nitroquinoline 1-Oxide by Microörganisms.

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4-Nitroquinoline 1-oxide has been known to be antimicrobial,[1,2] mutagenic,[3,4] carcinostatic[5] as well as carcinogenic.[6,7] Many investigations dealing with a series of quinoline 1-oxide derivatives showed that the nitro group at the 4-position in quinoline skeleton is indispensable for biological activities.[8,9]

A shift of ultraviolet absorption maximum of 4-nitroquinoline 1-oxide was observed when it was added to a washed cell suspension of *Candida utilis.*[10] The shift was inferred to be due to the modification of 4-nitroquinoline 1-oxide, probably in the nitro group. In the present work it is indicated that the shift in ultraviolet absorption spectra may be mainly due to the reduction of nitro group by microörganisms. Further it is shown that reduction of N→O bond at 1-position also occurs to a smaller extent.

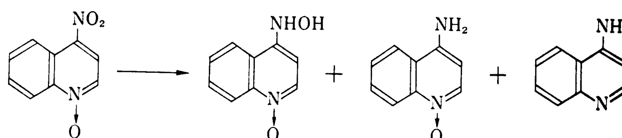

Chart 1.

Experimental

Materials and Methods

Bacterial Strains and Media——Microörganisms used were *E. coli* ATCC 9637,*2 *Brevibacterium liquefaciens*,[11] *Pseudomonas aeruginosa* IFO 3953,*3 *Candia utilis* OUT 6020*4 and *Aspergillus niger* W. For the conversion of 4-nitroquinoline 1-oxide by microörganisms the following media were used: Czapek's medium for *A. niger*, Hyduck's medium for *C. utilis*, glucose 2 g., KH_2PO_4 1.2 g., $Na_2HPO_4 \cdot 12H_2O$ 7.0 g., $(NH_4)_2SO_4$ 0.5 g., Na-citrate 0.5 g., $MgSO_4 \cdot 7H_2O$ 0.1 g. (per 1 liter of medium), pH 6.8 for *E. coli*; glucose 2 g., $(NH_4)_2HPO_4$ 1 g., $MgSO_4 \cdot 7H_2O$ 0.3 g., pH 7.0 for *Ps. aeruginosa;* sucrose 0.5 g., KH_2PO_4 3 g., $(NH_4)_2SO_4$ 1 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., pH 6.0 for *Brevibacterium liquefaciens.*

Paper Chromatography——One dimentional ascending paper chromatography was performed using the following solvent mixtures: iso-AmOH-Me_2CO-H_2O (4:2:1) and iso-AmOH-Me_2CO-H_2O (4:2:1)

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*4 Kindly supplied by Dr. Kyûbei Minoura of Department of Fermentation Technology, Osaka University, Osaka.

1) I. Arai, I. Nakayama : Yakugaku Zasshi, **72**, 167 (1952).
2) T. Okabayashi : Hakkokôgaku Zasshi, **31**, 373 (1953).
3) *Idem* : *Ibid.*, **33**, 513 (1955).
4) S. Mahima, Y. Ikeda : Appl. Microbiol., **6**, 45 (1958).
5) S. Sakai, K. Minoda, G. Saito, S. Akagi, A. Ueno, F. Fukuoka : Gann, **46**, 605 (1955).
6) W. Nakahara, F. Fukuoka, T. Sugimura : Gann, **48**, 129 (1957).
7) W. Nakahara, F. Fukuoka : Gann, **50**, 1 (1959).
8) T. Okabayashi : Yakugaku Zasshi, **73**, 946 (1953).
9) H. Endo : Gann, **49**, 151 (1958).
10) T. Okabayashi : Hakkokôgaku Zasshi, **32**, 108 (1954).
11) T. Okabayashi, E. Masuo : This Bulletin, **8**, 1089 (1960).

with NH_3 in a vapour phase. Location of spots were detected by an irradiation of ultraviolet light (2537 Å). Quinoline 1-oxides could be detected as fluorescent spots with various colors.
The other methods will be mentioned in each case.

Results

Transformation of 4-Nitroquinoline 1-Oxide by Microörganisms——As the first trial for this investigation, transformation of 4-nitroquinoline 1-oxide examined using several microörganisms. Cells of microörganisms grown in 100 cc. of appropriate media (broth, Hyduck and Czapek for bacteria, yeast and mould, respectively) were harvested by centrifugation, washed once with water and resuspended in 100 cc. of media described in experimental part.*5 Ten mg. of 4-nitroquinoline 1-oxide dissolved in a small amount of acetone was added, and the mixture was shaken for 3 hours at 28°. The suspension was centrifuged to sediment microbial cells. The ultraviolet spectra of a decimal dilution of the supernatants were measured with the Hitachi spectrophotometer using the decimal dilution of the uninoculated media as blank solutions. The results are shown in Fig. 1, which indicated that an exposure of 4-nitroquinoline 1-oxide to any one of the microörganisms cause a shift of ultraviolet absorption maximum of 4-nitroquinoline 1-oxide to shorter wave length. This may imply that all microörganisms tested have an ability to transform 4-nitroquinoline 1-oxide as was indicated by *C. utilis.*[10]

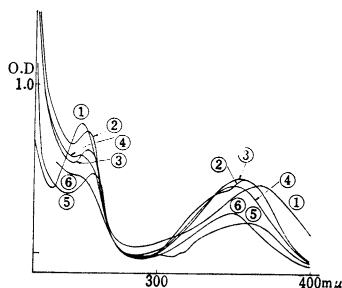

Fig. 1. Ultraviolet Absorption Spectra of Culture Fluids

1. 4-Nitroquinoline 1-oxide
2. *E. coli*
3. *Br. liquefaciens*
4. *Ps. aeruginosa*
5. *C. utilis*
6. *A. niger*

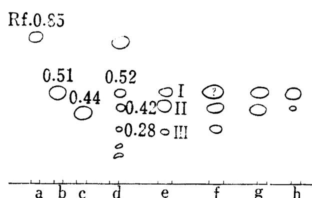

Fig. 2. Paper Chromatography of Ethanol Extracts

(a) 4-Nitroquinoline 1-oxide
(b) 4-Hydroxyaminoquinoline 1-oxide HCl
(c) 4-Aminoquinoline 1-oxide
(d) *Ps. aeruginosa*
(e) *E. coli*
(f) *C. utilis*
(g) *Br. liquefaciens*
(h) *A. niger*

The remainder of the supernatants were evaporated to dryness under reduced pressure, the residues were extracted with warm ethanol and the concentrates of the extracts were examined by paper chromatography.

As seen from the results indicated in Fig. 2, only the broth of *Ps. aeruginosa* contained 4-nitroquinoline 1-oxide (judged from paper chromatographic behavior and the fluorescent color similar to that of 4-nitroquinoline 1-oxide). In the extracts obtained from the broth of *E. coli*, *C. utilis*, and *Br. liquefaciens*, the spot of 4-nitroquinoline 1-oxide was disappeared and the substances with white blue fluorescence were mainly

*5 The amount of microbial cells used, except for *A. niger*, were determined from optical density at 500 mμ and the following values were obtained: *Ps aeruginosa*, 3.6; *E. coli*, 3.2; *C. utilis*, 4.0; *Br. liquefaciens*, 6.75. The amount of *A. niger* was 3.1 g. (wet weight).

given, showing Rf values of 0.50 (I) and 0.44 (II). In *A. niger*, (I) was a major product. Rf values of (I) and (II) were nearly as same as those of 4-hydroxyaminoquinoline 1-oxide and 4-aminoquinoline 1-oxide, respectively. The ratio of the yields of (I) and (II) judged from the intensity of fluorescence, was depending upon the microbial strains and cultural conditions employed.

In addition to the spot (I) and (II), *E. coli* and *C. utilis* gave faint but distinct spots giving Rf value of 0.24 with violet fluorescence (spot (III)).

Identification of (I) and (II) as 4-Hydroxyaminoquinoline 1-Oxide and 4-Aminoquinoline 1-Oxide——Six hundred cubic centimeters of broth medium was seeded with 20 cc. of inoculum culture of *E. coli* (shake cultured in broth medium at 28° for 20 hours). The cells (960 mg. in dry weight) were harvested by centrifugation, washed once with water and resuspended in 600 cc. of salt glucose medium (see experimental part). One hundred and twenty milligrams of 4-nitroquinoline 1-oxide was dissolved in a small amount of acetone and shaken on a rotary shaker for 3 hours at 28°. The broth was centrifuged to separate bacterial cells. An increase in bacterial mass to about twice of that of the original one was observed during incubation period. Supernatant was evaporated under reduced pressure to about 40 cc. and yellow precipitate was collected by centrifugation, washed with acetone and dried (66 mg. precipitate (I)). The residual supernatant was evaporated to dryness and the residue was extracted with warm ethanol. The extract was evaporated again to dryness, redissolved in a small amount of ethanol, and 200 cc. each of acetone and chloroform were added. The precipitate deposited was filtered off, and the solution was chromatographed through a florisil column (1×10 cm.). The substance retained in the column was eluted with the solvent described in Table I. Each fraction was evaporated to dryness and recovered as crude hydrochloric acid salt by redissolving in ethanol-hydrochloric acid and evaporating to dryness. Precipitate (I) was also converted to crude hydrochloric acid salt by the similar manner. The yield of crude hydrochloric acid salts are given in Table. I The data of paper chromatography of these salts were shown in Fig. 3, which indicated that precipitate (I) was the substance of spot (I) in Fig. 2 and that fraction 3 mainly contained the substance of spot (II).

Recrystallization of the crude hydrochloric acid salt of precipitate (I) from a mixture of methanol and ethyl acetate gave the crystals, m.p. 184~186° (decomp.), which were identified to be 4-hydroxyaminoquinoline 1-oxide hydrochloride by the mixed mel-

TABLE I. Chromatographic Separation of Transformation Products of 4-Nitroquinoline 1-oxide

Fraction	Solvents for elution		Crude HCl salt (mg.)
1	Acetone	200 cc.	2.0
2	Acetone-ethanol 90:10 v/v	200 cc.	8.9
3	Acetone-ethanol 80:20 v/v	200 cc.	27.1
4	Acetone-ethanol 70:30 v/v	200 cc.	7.0
5	Acetone-ethanol 50:50 v/v	200 cc.	4.6
6	Ethanol	200 cc.	7.5

TABLE II. Ultraviolet Absorption of I, II and III

	Wave length (mμ) of maximum absorption in	
	0.1*N* HCl	*M*/20 phosphate buffer pH 6.78
4-Hydroxyaminoquinoline 1-oxide	346, 248, 237, 217	360, 260, 220
I	346, 248, 237, 217	360, 260, 220
4-Aminoquinoline 1-oxide	347, 232, 213	358, 255, 217
II	347, 232, 213	358, 255, 217
4-Aminoquinoline	334, 322, 230	335, 322, 222
III	334, 322, 230	335, 322, 222

Table III. Paper Chromatography of I, II and III

	Rf with	
	iso-AmOH-Me_2CO-H_2O 4:2:1	iso-AmOH-Me_2CO-H_2O 4:2:1 (ammonia as vapor phase)
4-Nitroquinoline 1-oxide	0.84	0.82
4-Aminoquinoline 1-oxide HCl	0.49	0.49
4-Hydroxyaminoquinoline 1-oxide HCl	0.53	0.50[a] 0.20[b]
4-Aminoquinoline HCl	0.24	0.86
I HCl	0.52	0.49
II HCl	0.48	0.49 0.20
III HCl	0.24	0.86

a) Faint spot
b) Orange fluorescent spot. This may be due to the decomposed product of 4-hydroxyaminoquinoline 1-oxide.

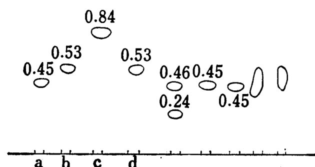

Fig. 3. Paper Chromatography of Fractions obtained from Column Chromatography

(a), (b), (c) : The same as Fig. 2
(d) : Precipitate I

ting point, infrared spectrum, ultraviolet spectra in acid and neutral buffer solutions (Table II), and paper chromatographic behavior (Table III).

Purified sample of fraction 3 by recrystallization from a mixture of ethanol and ethyl acetate was established to be 4-aminoquinoline 1-oxide (Table II and III).

Identification of spot (III) as 4-aminoquinoline——Fraction 2 in Table I gave two fluorescent spots by irradiation of paper chromatogram by ultraviolet light. The substance moving faster was thought to be 4-aminoquinoline 1-oxide, judged from the mobility on paper and the color of fluorescence. The substance moving slower gave a violet fluorescence with Rf value of 0.25. This substance was separated by preparative paper chromatography on filter paper (40×20 cm.), Tôyô Roshi No. 53. The band corresponding to this substance was cut out and eluted with 70% ethanol. The eluate was acidified with an addition of a small amount of *N*-hydrochloric acid and evaporated to dryness. Colorless needles were obtained and the yield was 7 mg. per 2.1 L. of suspending broth. In comparing the properties of this substance with those of 4-aminoquinoline-hydrochloride, both were found to be identical (Tables II, III and Fig. 4).

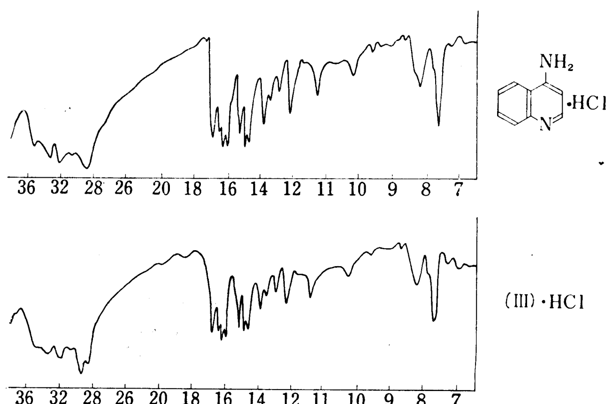

Fig. 4. Infrared Spectra of 4-Aminoquinoline and III

Discussion

The results from our investigation show that the reduction of 4-nitroquinoline 1-oxide proceeds as depicted in Chart 1. It is reminiscent of the catalytic reduction of 4-nitroquinoline 1-oxide as reported by Ochiai and Naito.[12] It is noteworthy that the N→O bond at 1-position was also resistant to microbial reduction as indicated in catalytic reduction. From the results indicated in Fig. 2, it seems reasonable to assume that the reduction of 4-nitroquinoline 1-oxide as presented in Chart 1 is a common phenomenon with microorganisms.

Although many nitro compounds have been reported to manifest a variety of biological activities, none are known to have mutagenic or carcinogenic activities as powerful as 4-nitroquinoline 1-oxide. The importance of nitro group in the biological activities of nitroquinoline 1-oxides was deduced from the following facts : 1) In a series of nitroquinoline 1-oxide derivatives a close relationship seems to exist between the chemical activity of the nitro group and their biological activities.[9,10,13] 2) The nitro group can easily be substituted by SH compounds nonenzymatically in physiological conditions.[8] 3) The content of SH compounds in skin epidermis rapidly decreased when 4-nitroquinoline 1-oxide was applied to the skin of mice.[14]

Above facts, together with various observations that have been made on the biological action of 4-nitroquinoline 1-oxide, unequivocally suggested that the nitro group in this agent exert peculiar effects on living matter. Moreover, it was inferred that the substitution reaction of nitro group with SH compounds is the primary reaction of 4-nitroquinoline 1-oxide in the manifestation of its biological activities. Therefore it was rather unexpected that the nitro group at 4-position can be reduced by microoganisms easily as reported in a variety of organic nitro compounds using microoganisms and animals.[15~18]

Alternatively it is interesting that the 4-hydroxyamino compound, a possible intermediate in the biological reduction of aromatic nitro compounds accumulated in the broth of microoganisms in large amount. Although there have been many investigations dealing with a reduction of aromatic nitro compounds with a variety of biological systems, a comparatively small amount of information has been obtained on hydroxyamino compounds.[19~21] Therefore 4-nitroquinoline 1-oxide may be applicable as a useful tool to the investigation of the biological reduction of aromatic nitro compounds.

It is also interesting to know that the reduction of 4-nitroquinoline 1-oxide seems to vary depending on the microoganism. For example *A. niger*, which is most sensitive to 4-nitroquinoline 1-oxide and easily mutated by the treatment of this agent, could reduce it only to 4-hydroxyaminoquinoline-1-oxide, while *E. coli*, on which 4-nitroquinoline 1-oxide has only a slight mutagenic effect, gave additional reduction products.

Now attempts are being made to persue the reduction process using newly established colorimetric methods.[22] The results obtained from these studies are consonant with

12) E. Ochiai, T. Naito : Yakugaku Zasshi, **64**, 206 (1944).
13) T. Okabayashi : Hakkokôgaku Zasshi, **35**, 17 (1957).
14) Y. Hayashi : Gann, **50**, 219 (1959).
15) R.B. Cain : Biochem. J., **73**, 305 (1959).
16) A.K. Saz, R.B. Slie : Arch. Biochem. Biophys., **51**, 5 (1954).
17) R.T. Williams : "Detoxication mechanism" second ed. 410 (1959). John Wiley & Sons Inc. New York.
18) J.R. Fouts, B.B. Brodie : J. Pharmacol., **119**, 197 (1957).
19) H.J. Chanon, G.T. Millis, R.T. Williams : Biochem. J., **38**, 70 (1944).
20) M. Zucker, A. Nason : "Methods in Enzymology" **2**, 406 (1955). Academic Press, New York.
21) I. Yamashina, S. Shikata, F. Egami : Bull, Chem. Soc. Japan, **27**, 42 (1954).
22) T. Okabayashi, A. Yoshimoto : unpublibhed data.

the qualitative results presented here. Recently in a separate experiment, it was found that the microbial reduction described here has a very important significance in the manifestation of mutagenic activity by 4-nitroquinoline 1-oxide. A preliminary account on these findings has already been published[23]

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Summary

The ultraviolet absorption maximum of 4-nitroquinoline 1-oxide was shifted toward shorter wave length when the substance was added to the growing media of microorganisms. The investigation of this phenomenon revealed that the shift in ultraviolet absorption maxima is due to the reduction of nitro group at 4-position. It was also found that the reduction of N→O bond in 1- position occurs to a smaller extent.

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23) T. Okabayashi : This Bulletin, 10, 1127 (1962).

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203. **Ryuichi Kimura, Takahiro Yabuuchi, Masakatsu Hisaki, Hideki Sugimoto, Akio Ohyama, and Kooichi Mochida** : Studies on the Synthesis of New Antimicrobials. I. Synthesis of 2,5-Dichloroaniline Derivatives and their Some Antibacterial Activities.

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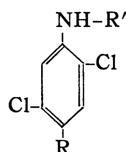
The antimicrobacterial activity of dichloroaniline has been of great interest for several years. In 1957, 2,5-dichloro-4-thiocyanatoaniline was synthesized by Yoshina,[1] and its potential antibacterial and antifungal activities were investigated.[2] Particularly 2,5-dichloro-4-thiocyanatoaniline was found to inhibit the growth of Tricophyton at a concentration of 3 γ/ml. Recently, several derivatives of halogenated benzene, such as pentachlorophenol, Bithionol etc., hove been used widely as a marked disinfectant.

Authors attempted to synthesize a number of N-amide derivatives of 2,5-dichloro-, 2,5-dichloro-4-bromo-, and 2,5-dichloro-4-thiocyanatoaniline.

The general structure is shown as follows.



R : -H, -Br or -SCN
R′ : various groups.

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1) S. Yoshina : Japan Patent, 18567 (1960), Dec. 23.
2) T. Tokunaga : The Bulletin of the Fukuoka Medical School, 49, No. 9 (1957). A. Ohyama, *et al.*: The Bulletin of the Japan Society of Chemotherapy, 9, 329 (1961).