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37. Tadashi Okabayashi and Akihiro Yoshimoto : Colorimetric Determination of 4-Hydroxyaminoquinoline 1-Oxide and 4-Aminoquinoline 1-Oxide.

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In the preceding paper we demonstrated that 4-hydroxyaminoquinoline 1-oxide (4HAQO) is mutagenic on *Aspergillus niger*.[1] Very recently it was indicated that 4HAQO caused the bacteriophage induction in lysogenic bacteria.[2] It was also reported that 4HAQO has carcinogenic activity as powerful as 4-nitroquinoline 1-oxide (4NQO).[3] These facts may involve the possibility that 4HAQO is the proximate mutagenic (or carcinogenic) substance after the exposure (or administration) of 4NQO.[*2]

We could demonstrate that nitro group of 4NQO is rapidly reduced by various microorganisms. Examination by paper chromatography and the isolation of reduction products revealed the accumulation of considerable amount of 4HAQO in culture broth of some microorganisms.[4] From these facts the biological reduction of 4NQO is of significance from mutagenic (or carcinogenic) activity. It became desirable, therefore, to carry out more detailed study on the biological reduction of 4NQO.

One of the difficulties hampered this study is that no suitable method has been established to measure the reduction products. For instance our preliminary experiment to determine 4-aminoquinoline 1-oxide (4AQO) by the method of Bratton and Marshall,[5] which is widely used for the determination of arylamines, was unsuccessful because the method gave only feeble and unstable coloration of aminoazo dye for 4AQO.

The present investigation was designed to overcome this problem. As will be indicated later we succeeded in estimating 4HAQO and 4AQO, the major microbial reduction products of 4NQO. Further, some informations on the microbial reduction of 4NQO were obtained by using this method.

Materials and Methods

Chemicals——4HAQO·HCl was supplied by Dr. Masakazu Hamana, Professor of Kyushu University and Ryōzō Maeda of our laboratory. 4AQO·HCl was supplied by Dr. Okamoto, Professor of Tokyo University. 4NQO was synthesized by method of Ochiai.[6] Pentacyanoamine ferroate was prepared by the method of Zucker and Nason.[7] Other chemicals used in this work were obtained from commercial sources.

Reagents——For the estimation of 4HAQO and 4AQO following reagents were prepared. i) 0.2% $NaNO_2$; ii) conc. (12*N*) HCl; iii) 0.5% ammonium sulfamate; iv) 0.1% 2-hydroxy-6,8-naphthalenedisulfonic acid (G-acid), dissolved in minimal amount of NaOH; v) 40% KOH; vi) 0.5% pentacyanoamine ferroate. Reagents (i, iv and vi) were freshly prepared for each determination.

Measurement of 4HAQO——Procedure used for the estimation of 4HAQO was a modification of Zucker and Nason[7] developed for the assay of *m*-nitrophenylhydroxylamine. To 3 ml. solutions containing 20 to 150 μg. of 4HAQO (as HCl salt), 2 ml. of pentacyanoamine ferroate reagent were added.

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*2 It should be noted that 4HAQO does not have vesicant action to the skin, which is one of the most characteristic properties of 4NQO. This difference between 4NQO and 4HAQO was briefly discussed.[1]

1) T. Okabayashi, A. Yoshimoto, M. Ide : This Bulletin, **12**, 257 (1964).
2) H. Endo : Private communication.
3) Y. Shirasu, A. Ohta : Gann, **54**, 221 (1963).
4) T. Okabayashi, A. Yoshimoto : This Bulletin, **10**, 1221 (1962).
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6) E. Ochiai : J. Org. Chem., **18**, 534 (1953).
7) M. Zucker, A. Nason : "Methods in Enzymology," **2**, 406 (1955). Academic Press Inc., New York.

The mixtures were allowed to stand at 37° for 1 hr., after which coloration (orange) was measured against 0.5% pentacyanoamine ferroate solution at 520 mμ by using a Hitachi spectrophotometer.

Determination of 4AQO——The present method is besed on the finding of Ochiai and Naito,[8] and Ochiai and Teshigawara,[9] who demonstrated that diazotized 4-aminopyridine 1-oxide and 4AQO, after coupling with phenols in alkaline conditions, gave red or purple hydroxyazo dyes. After the survey of coupling agents, we found that G-acid is one of the most preferable reagents as β-component, and developed the procedure as outlined below. To 1 ml. of solutions containing 10 to 80 μg. (as HCl salt) of 4AQO, 0.3 ml. of concentrated HCl and 0.25 ml. of $NaNO_2$ solution were mixed. The mixtures were allowed to stand for 20 min. at room temperature, after which excess $NaNO_2$ was destroyed by the addition of 0.5 ml. of ammonium sulfamate solution. After standing for 10 min., 0.5 ml. of G-acid solution and 1 ml. of 40% KOH was added, then the mixture was made up to 10 ml. with H_2O. The blue color, which developed immediately was measured at 590 mμ against a blank solution which was treated similarly as samples, except that 1 ml. of H_2O was used in place of 4AQO solution.

Microbial Strains and Media——Microorganisms used were *Escherichia coli* ATCC 9637 and *A. niger* W. The culture media and the cultural conditions were the same as those already been reported.[4] For the preparation of washed cell suspension of *E. coli*, the culture broth (shake cultured for 18 hr. at 28°) was centrifuged. The cells were harvested, washed twice with 0.062*M* phosphate buffer (pH 7.2) and resuspended in a small amount of the same buffer. The final cell density in reaction mixture was adjusted as described in Fig. 5.

The washed mycelia of *A. niger* were prepared by filtration of shake cultured broth with a cloth and washing with the phosphate buffer.

Results

Determination of 4HAQO

Fig. 1 shows an example of calibration curve obtained for 4HAQO. Although the absorbancy of colored complex was greater in shorter wave lengths (480 mμ or 460 mμ) we determined the color intensity at 520 mμ to minimize the erroneous results caused from the absorbancy of blank solutions (pentacyanoamine ferroate), which increased sharply in shorter wave length than 520 mμ.

As indicated in the figure coloration was rather insensitive, and the linear response was obtained only within narrow range of 4HAQO concentration. Therefore attempts were made to estimate 4HAQO by several methods which based on different principles from the present method. All these attempts were unsuccessful. These include the

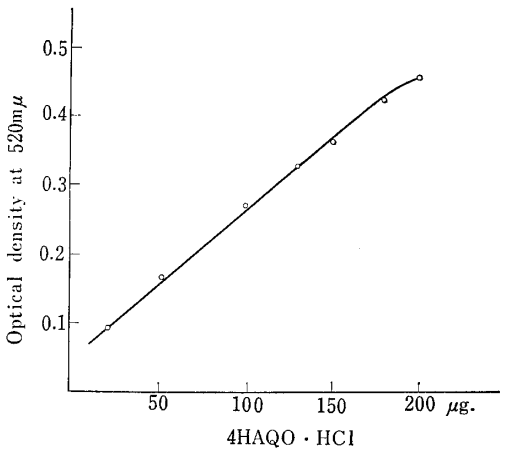

Fig. 1. Calibration Curve for 4-Hydroxyaminoquinoline 1-Oxide (4HAQO)

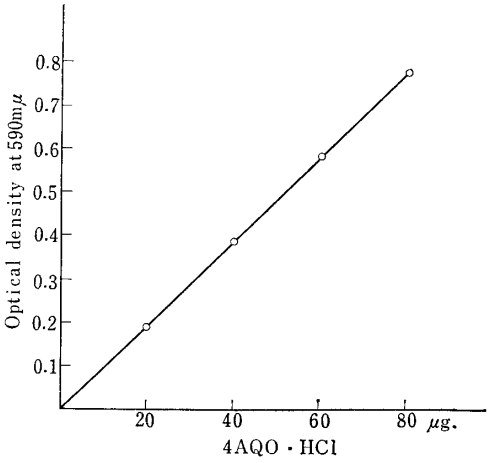

Fig. 2. Calibration Curve for 4-Aminoquinoline 1-Oxide (4AQO)

8) E. Ochiai, T. Naito : Yakugaku Zasshi, **65**, 441 (1945).
9) E. Ochiai, T. Teshigawara : *Ibid.*, **65**, 435 (1945).

coloration of hydroxyamino compounds after the reduction of alkaline triphenyltetrazolium chloride,[10,11] and the coloration with Ehrlich's reagent.[12]

In the condition described above, coexistence of 4NQO and 4AQO (300 μg. each) did not appreciably affect the coloration. However, calibration curve varied in different experiments, so it is desirable to set up standards containing known amounts of 4HAQO, in each determination.

Determination of 4AQO

Fig. 2 shows an example of calibration curve obtained for 4AQO. The coloration was stable for at least several hours, and showed a linear response over the range 0 to 80 μg. The presence of 4NQO and 4HAQO did not affect the coloration.

One of the difficulties in colorimetric estimation of 4AQO was that it was resistant to diazotization. The conditions used in usual arylamine determinations were insufficient and more drastic condition was needed. Diazotization in concentrated H_2SO_4 in ice cold bath[8,9] gave intensive coloration after coupling with G-acid. However, the color intensity of the resultant hydroxyazo dye fluctuated to a considerable extent. After the survey of condition we found that diazotization in hydrochloric acid at room temperature was the most preferable. The time course of this reaction was followed by absorption at 590 mμ (Fig. 3). It was found that the reaction reaches to plateau after 20 to 30 minutes. The presence of 2.3 to 3.5*M* HCl is also indispensable (Fig. 4).

One of the disadvantages in the present method is that the existence of organic solvents such as acetone or ethanol caused the decrease in color intensity. Therefore in routine estimation of 4AQO, caution was made to avoid the use of organic solvents as far as possible. Another inconvenience is that calibration curve differed slightly in different experiments, therefore we set standard tubes containing known amounts of 4AQO in each determination.

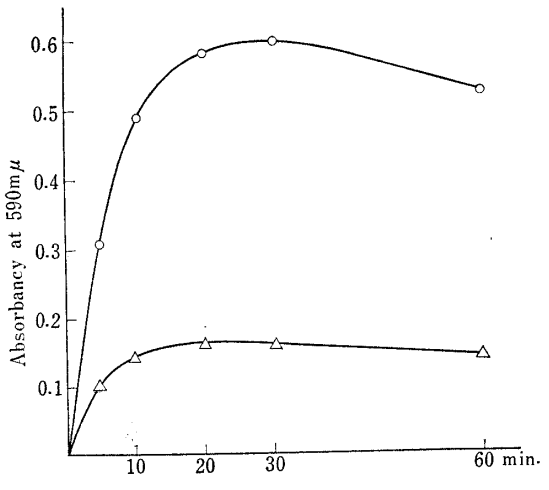

Fig. 3. Effect of Time on Diazotization

4AQO·HCl solution (0.5 ml.) was added by 0.5 ml. of 1:1 HCl and 0.25 ml. of $NaNO_2$. Diazotization was carried out at 25°.
—○—○— 60 μg. —△—△— 20 μg.

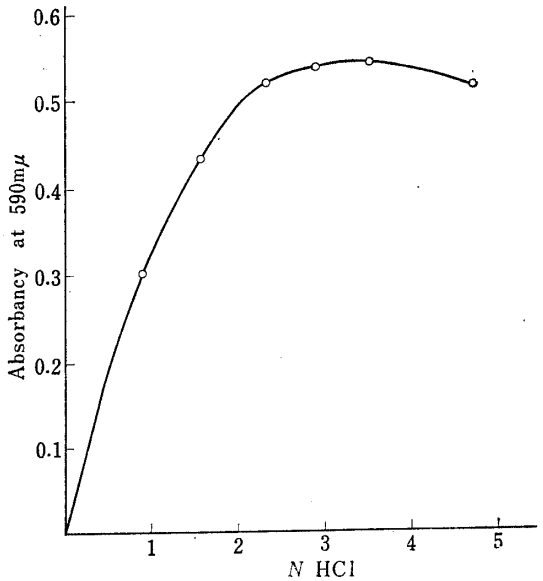

Fig. 4. Effect of Concentration of Hydrochloric Acid

4AQO·HCl: 60 μg.

10) E. Ochiai, A. Ohta, H. Nomura : This Bulletin, **5**, 310 (1957).
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12) N. J. Cartwright, R. B. Cain : Biochem. J., **73**, 305 (1959).

Reduction of 4NQO by Washed Cell Suspensions of Microorganisms

As the application of the present methods the reduction of 4NQO by washed cell suspensions of *E. coli* or *A. niger* was studied. To 90 ml. of 0.55 m*M* of 4NQO in 0.062*M* phosphate buffer (pH 7.2) 10 ml. of washed cell suspensions were added. The incubation was performed at 28° under shaking (aerobic) or bubbling nitrogen gas (anaerobic). Samples were withdrawn at intervals, chilled immediately by dipping in an ice bath, and centrifuged at 2°. In case where the supernatant remained cloudy it was cleared by Seitz filtration. The contents of 4HAQO and 4AQO in the supernatants were estimated as described above.

For the estimation of residual 4NQO, 5 ml. of the supernatant were acidified by the addition of H_2SO_4 and extracted three times with 5 ml. of chloroform. The extracts were combined, and dried over anhyd. sodium sulfate. 4NQO was estimated from absorbance at 390 mμ and the molecular extinction coefficient of 14,800.

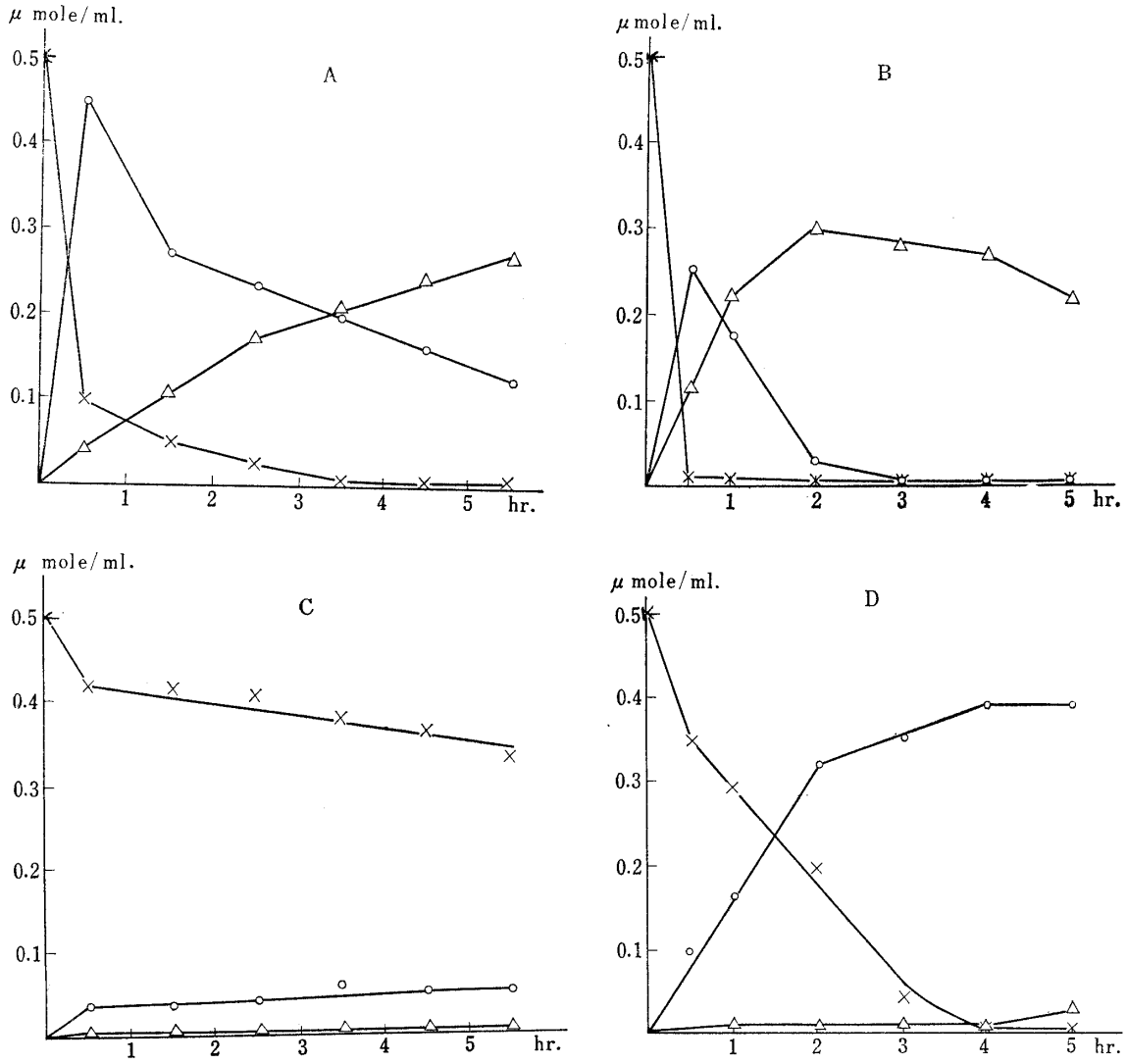

Fig. 5. Reduction of 4-Nitroquinoline 1-Oxide by Washed Cell Suspensions of *E. coli*

Each reaction mixture contains 0.062*M* phosphate buffer (pH 7.2), with or without glucose (0.2%), washed cell suspension of *E. coli* (absorbance at 500 mμ=3.3) and 5×10^{-7} mol./ml. of 4NQO.
A. aerobic condition in the presence of glucose
B. anaerobic condition in the presence of glucose
C. aerobic condition in the absence of glucose
D. anaerobic condition in the absence of glucose
—×—×— 4NQO —○—○— 4HAQO —△—△— 4AQO

Fig. 5A shows the reduction of 4NQO by *E. coli*. Incubation was carried out aerobically in the presence of 0.2% glucose. 4AQO decreased rapidly and completely disappeared after about 3 hours' incubation. 4HAQO increased rapidly and then decreased gradually with a concomitant increase of 4AQO.

The reduction of 4NQO in anaerobic condition occurred so rapidly that disappearance of yellow color of 4NQO was observed visually within 5 minutes. After 30 minutes 4NQO completely disappeared and considerable amount of 4AQO had already appeared (Fig. 5B). It is notable that longer incubation (more than 3 hours.) caused the decrease of 4AQO. Examination of the suspension by paper chromatography with methyl ethyl ketone-*sec*-butanol-water*[3,13] showed the increase of 4-aminoquinoline (4AQ). Thus the decrease of 4AQO in the later stage of incubation might be explained by further reduction of 4AQO to 4AQ.

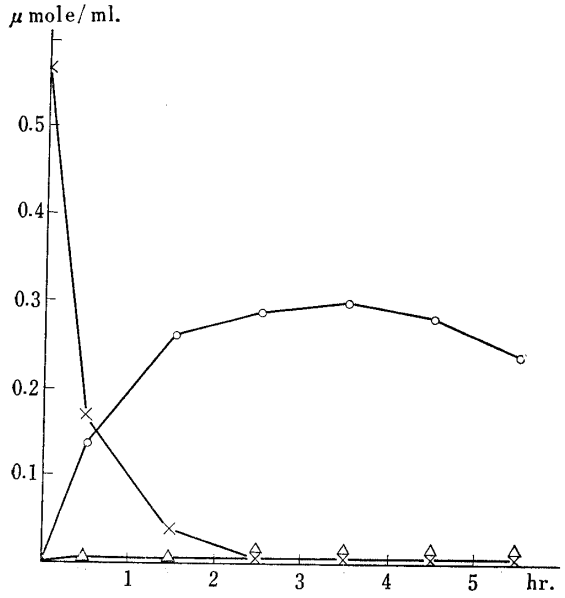

Fig. 6. Reduction of 4-Nitroquinoline 1-Oxide by Washed Mycelia of *A. niger*

The conditions are the same as Fig. 5 A except that 3.75 g. (wet wt.) of washed mycelia were used.

—×—×— 4NQO
—○—○— 4HAQO
—△—△— 4AQO

As depicted in Fig. 5C, the reduction of 4NQO was markedly hindered by exclusion of glucose from reaction mixture. In this case only slight decrease of 4NQO and the formation of approximately the corresponding amount of 4HAQO was observed. The same experiment performed in an atmosphere of nitrogen obtained more prominent reduction of 4NQO, and was reached accumulation of up to about 80% of originally added 4NQO (Fig. 5D). The formation of small amount of 4AQO was also observed after four hours' incubation.

Fig. 6 shows the results obtained with the same condition as employed in Fig. 5A by use of *A. niger*. In this experiment 3.75 g. of wet mycelia per 100 ml. of reaction mixture were used in stead of *E. coli* suspension. Although rapid decrease of 4NQO and formation of 4HAQO (correspond to 60% of originally added 4NQO) were observed as to be expected from the case of *E. coli* (Fig. 5), no appreciable amount of 4AQO was formed.

Discussion

Although many investigations have dealt with biological reduction of aromatic nitro compounds, the determination methods of reduction products used in these studies cannot be applied for the reduction products of 4NQO without modification. The present methods, though a little troublesome and insufficient with regard to accuracy in the determination of 4HAQO, may give the first basis for the quantitative treatment in the biological reduction of 4NQO and its related compounds.

The microbial reduction of 4NQO studied by using the present methods is consistent with the results obtained by visual inspection of paper chromatogram, carried out in

*3 By the reason which is obscure at the present time, the use of the solvent system used in a previous investigation[4] (iso-AmOH-Me_2CO-H_2O) sometimes caused overlapping of 4HAQO and 4AQO. So EtCOMe-*sec*-BuOH-H_2O was used in this work. By use of this solvent system we obtained good separation of 4NQO, 4HAQO, 4AQO and 4AQ.

13) T. Okabayashi, M. Ide : J. Chromatog., **9**, 523 (1962).

parallel with the colorimetric determination. One characteristic feature in the present experiment is that a large amount of 4HAQO accumulated in the course of reduction. Although many investigations have revealed that hydroxyamino compounds are intermediates in the reduction of arylnitro compounds, the survey of literatures did not uncover the accumulation such a large amount of hydroxyamino compounds as indicated in this experiment. As discussed earlier,[4] the biological activities of nitroquinoline 1-oxides are rather peculiar among diverse organic nitro compounds. This peculiarity may, if not all but at least partly, be explained by the accumulation of large amount of hydroxyamino intermediates in the course of metabolism.

We wish to thank Prof. Emeritus Eiji Ochiai, University of Tokyo for his encouragement. This work was partially supported from the Ministry of Education of Japan.

Summary

Colorimetric methods for the estimation of 4-hydroxyaminoquinoline 1-oxide and 4-aminoquinoline 1-oxide have been developed. The former was estimated by a modification of Zucker and Nason's method (Zucker and Nason, 1955). The method for the estimation of 4-aminoquinoline 1-oxide is based on the formation of a stable hydroxyazo dye which is formed after coupling of diazotized 4-aminoquinoline 1-oxide with 2-hydroxy-6,8-naphthalenedisulfonic acid in alkaline condition.

The formation of 4-hydroxyaminoquinoline 1-oxide and 4-aminoquinoline 1-oxide in the course of microbial reduction of 4-nitroquinoline 1-oxide was studied by using the present methods.

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UDC 612.398.145

38. Morio Ikehara, Hitoshi Uno, and Fumiyoshi Ishikawa : Studies of Nucleosides and Nucleotides. XXIII.*1 A Versatile Method for Replacement of 6-Hydroxyl Group of Purine Nucleoside.

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In the previous papers from our laboratory[1~3] the synthesis of purine nucleoside and nucleotide bearing various substituents in 6-position has been reported. In the main, 6-hydroxyl group was converted to mercapto group by the reaction with phosphorus pentasulfide in refluxing pyridine[4] and then replaced by the attack of various nucleophiles. Another course investigated so far by several researchers[5~8] involved

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*2 Kita 12-jo, Nishi-5-chome, Sapporo (池原森男, 宇野 準, 石川文義).

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7) R. K. Robins, *et al.* : J. Am. Chem. Soc., **82**, 2654 (1960); J. Org. Chem., **26**, 477 (1961); *Ibid.*, **27**, 986 (1962).
8) H. Lettlré, H. Bullweg : Ann., **656**, 158 (1962).