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Studies on Chemical Carcinogens. IX.[1] Homolytic Degradation of O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide (1-Acetoxy-4-acetyloxyimino-1,4-dihydroquinoline)

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O, O′-Diacetyl-4-hydroxyaminoquinoline 1-oxide was found easily to liberate a considerable amount of acetic acid in inert organic solvents such as dioxane as well as in crystalline form. The acetic acid formation was regarded as being initiated by homolytic fission of N–O bond of the substituent hydroxyamino group and studied with a help of electron spin resonance spectroscopy. A brief discussion was made of the correlation of free radical formation with carcinogenesis of carcinogenic hydroxylamine derivatives.

There have been found some kinds of arylhydroxylamines which have potent ability in tumor induction at the site of subcutaneous injection in mice and rats. 4-Hydroxyaminoquinoline 1-oxide,[3,4] N-hydroxy-2-acetylaminofluorene,[5–7] and 4-(N-methyl-O-benzoyl)-hydroxyaminoazobenzene[8,9] are the typical examples which can be considered as the proximate carcinogens of 4-nitroquinoline 1-oxide, 2-acetylaminofluorene, and 4-dimethylaminoazobenzene, respectively. Recently, we found that acetyl derivative of 4-hydroxyaminoquinoline 1-oxide, formulated as O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide (I),[10] showed strong carcinogenic effect on mice.[4] This diacetyl derivative (I) is much more labile than the parent 4-hydroxyaminoquinoline 1-oxide[10] and it may be an interesting compound in connection with the carcinogenesis of other types of acylated hydroxylamines mentioned above.

This paper describes chemical property of this diacetate, especially in relation to the radical formation. Preliminary results are also reported on the electron spin resonance study of some related acylhydroxylamines in connection with their carcinogenic activity.

Results

Chemical Properties

O,O′-Diacetyl-4-hydroxyaminoquinoline 1-oxide (I) is soluble in ether, chloroform, dioxane, and other conventional organic solvents but almost insoluble in water. It is conveniently recrystallized from ether to form colorless needles, melting at 110°. During storage

1) Part VIII: Y. Kawazoe, M. Araki (née Tachibana), and W. Nakahara, *Chem. Pharm. Bull.* (Tokyo), **17**, 544 (1969).
2) Location: *Tsukiji, Chuoku, Tokyo.*
3) Y. Kawazoe, M. Tachibana, K. Aoki, and W. Nakahara, *Biochem. Pharmacol.*, **16**, 631 (1967) and literatures cited therein.
4) Y. Kawazoe, M. Araki, and W. Nakahara, *Chem. Pharm. Bull.* (Tokyo), **17**, 544 (1969).
5) J.W. Cramer, J.A. Miller, and E.C. Miller, *J. Biochem.*, **235**, 885 (1960).
6) J.A. Miller, J.W. Cramer, and E.C. Miller, *Cancer Res.*, **20**, 950 (1960).
7) E.C. Miller, J.A. Miller, and H.A. Hartmann, *Cancer Res.*, **21**, 816 (1961).
8) K.Sato, L.A. Poirier, J.A. Miller, and E.C. Miller, *Cancer Res.*, **26**, 1678 (1966).
9) L.A. Poirier, J.A. Miller, E.C. Miller, and K. Sato, *Cancer. Res.*, **27**, 1600 (1967).
10) Y.Kawazoe and M. Araki, *Gann*, **58**, 485 (1967).

of this diacetate in crystalline form, acetic acid was gradually evolved to leave unidentified decomposed products unless it was stored in a well-degassed desiccator over phosphorous pentaoxide. It is interesting to note that such ready evolution of acetic acid took place when dissolved in organic solvents, too. This kind of acetates can be expected, in general, to be a potent acetyl donor to -NH, -OH, *etc.* As a matter of fact, when the diacetate reacted with aniline in pyridine, acetanilide was formed indeed, but the yield of 26% was much poorer than the expected. The same situation was observed when alcohols were used as an acetyl acceptor instead of amines. When this diacetate was hydrolyzed by warming its aqueous suspension in various pH solvents (pH: 5.0, 7.0, and 8.0), decomposition of the diacetate was readily completed in either pH solvent but the recovery of 4-hydroxyaminoquinoline 1-oxide could not definitely be observed among the decomposition products by thin-layer chromatography.

These experimental results may strongly suggest that, in addition to the ionic acetyl migration, this diacetate undergoes free radical reactions, starting with a homolytic fission of N–O bonds in the molecule, as illustrated in Chart 1.

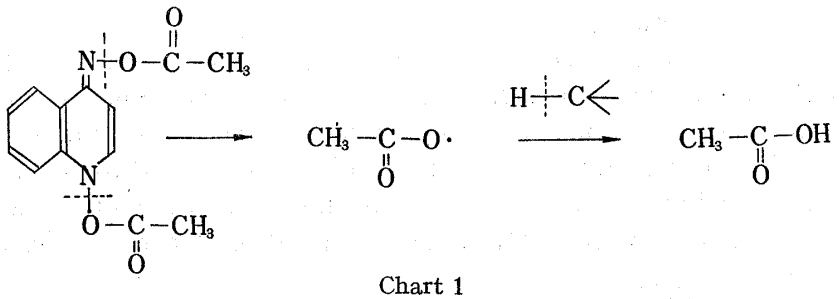

Chart 1

The degradation process of this diacetate was examined in dioxane, isopropanol, *tert*-butanol, methanol, and aqueous solutions. A half mmole (130 mg) of the diacetate was dissolved in 1 to 2 ml of dioxane dried over sodium wire in a sealed tube and warmed at 70° for 2.5 hr. The reaction mixture was deeply colored. The volatile component of the reaction mixture was completely collected by vacuum distillation. The distillate was diluted with *ca.* 2 ml of water and titrated with 0.1N sodium hydroxide solution. The result indicated that 0.6 equivalent mole of acetic acid had been liberated by warming the diacetate in dioxane. This was also confirmed by nuclear magnetic resonance spectroscopy. A subsequent same treatment of the residue of the volatile distillate gave another 0.4 to 0.5 equivalent mole of acetic acid. As a result, 0.9 to 1.0 equivalent mole of acetic acid was obtained in total. The similar experiment was carried out using isopropanol as the solvent. The titration of the volatile component of the reaction mixture indicated that 1.0 equivalent mole of acetic acid was liberated. Then, the solution thus titrated was made alkaline by addition of a definite amount of sodium hydroxide solution and warmed at 70° for 2 hr. The reaction mixture was titrated with 0.1N hydrochloric acid, showing that further 0.4 equivalent mole of acetic acid was liberated, which had been produced by alkaline hydrolysis of isopropylacetate. This was also confirmed by gas chromatographic analysis of the volatile component of the reaction mixture. When methanol was used as the reaction solvent, 0.3 equivalent mole of acetic acid was evolved but no appreciable amount of methyl acetate was detected under the same reaction condition. The acetic acid–formation was observed even in *tert*-butanol in a relatively high yield, although this may be rather surprising when one would realize the homolytic abstruction of hydrogen from the solvent molecule. In any way, it is sure that the acetoxy free radical formed by homolytic fission of the N–O bond abstructs a hydrogen from solvent or substrate molecules to form acetic acid, as illustrated in Chart 1. The results are summarized in Table I. As seen in the table, the fact that 1 mole or less of acetic acid was evolved in either case may indicate that one of the two acetoxy groups in the molecule may be split into an acetoxy free radical, $CH_3COO\cdot$, as the initiation of the degradation of this

Table I. Formation of Acetic Acid and Acetates by Warming the O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide in Various Solvents at 70° for Several Hours

Solvent	Acetic acid[a] (eq. mole)	Acetate[a] (eq. mole)
Water	1.7	.....
Methanol	0.4	a trace
Isopropanol	0.9	0.4
tert-Butanol	0.9	0.3
Dioxane	0.9	.....

a) Reproducibility was not very good in either case.

diacetate. The same treatment of the aqueous suspension of the diacetate gave 1.7 equivalent mole of acetic acid. It may be plausible that, in aqueous and alcoholic solvents, ionic acetyl transfer from the diacetate to the OH group of the solvent took place in addition to free radical reactions.

When the nuclear magnetic resonance spectral change was traced with time in dioxane solution, two of the acetyl signals decreased in intensity to the same extent, while another acetyl signal due to acetic acid appeared and grew more intense. This may suggest that the residual moiety having liberated one acetoxy radical is not stabilized to undergo facile further degradations such as polymerization. It is to be noted, furthermore, that no detectable amount of gaseous products were evolved during the degradation in dioxane.

Electron Spin Resonance Study

Since the chemical data revealed that this degradation involved free radical reaction, the radical thus produced was studied by electron spin resonance spectroscopy.

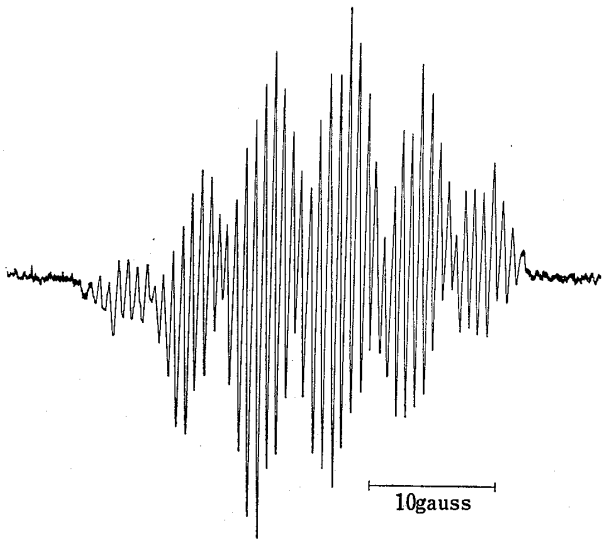

Fig. 1. Electron Spin Resonance Spectrum of the Free Radical Produced from O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide in Dioxane, Modulation Width being 0.7 gauss

When the diacetate dissolved in dioxane was warmed at about 70°—80° for a short period of time, intense electron spin resonance signal was observed at room temperature, as shown in Fig. 1. The free radical thus produced seemed considerably stable and could be stored in a deep freezer (*ca.* −20°) for several days without any signal attenuation. The signal was split into sixtet when measured with 4.0 gauss modulation and split into forty-eight lines with 0.7 gauss modulation, the smallest coupling constant being 0.74 gauss. It is worth noting that the induction period was evidently proved for radical production since very intense signal was found with a few mg of the diacetate even at room temperature by addition of a trace of the pre-decomposed products from the diacetate.

In order to determine the free radical structure, the electron spin resonance spectrum was analyzed with a help of the derivatives replaced with ^{15}N ($I_{^{15}N}=1/2$; $\gamma_{^{15}N}/\gamma_{^{14}N}=1.403$[11])

11) The change in coupling constant induced by replacing ^{14}N and H with ^{15}N and D, respectively, is proportional to the relative ratio of the magnetogyric ratio (γ) of the isotopes.

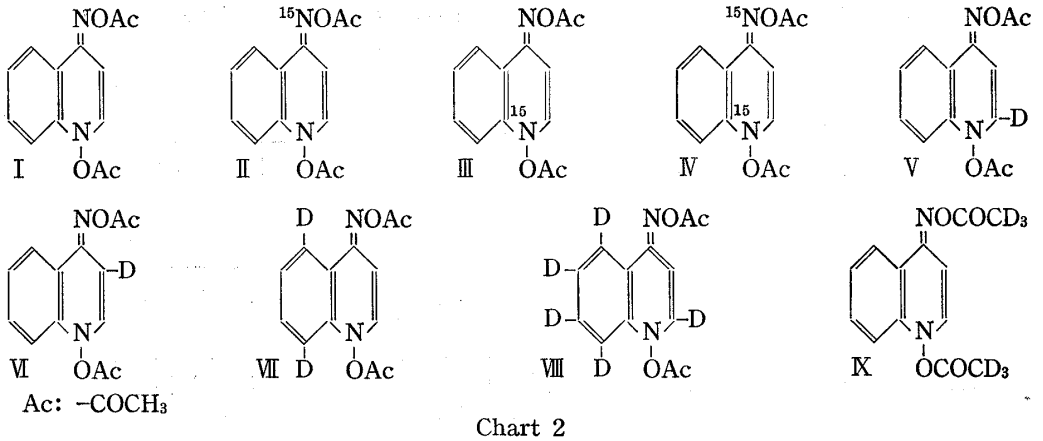

Chart 2

or D ($I_D=1$; $\gamma_D/\gamma_H=0.154$[11]).[12-14] The compounds used for this purpose are shown in Chart 2 and their electron spin resonance spectra measured in dioxane with 4 gauss modulation are reproduced in Fig. 2 and 4.

The equally split sixtet of I was deformed into an equally split quartet by replacement of both nitrogens with ^{15}N nucleus, the spacing shifting from 5.70 to 7.21 gauss. Mono-^{15}N-replaced derivatives, II and III, gave quintet signals, the spacing being 6.25 and 6.13 gauss, respectively. These facts indicates that the sixtet splitting of the non-isotopic derivative is attributed to the spin-couplings with two nitrogen nuclei (I=1) and one proton (I=1/2) wit the almost same magnitude of the coupling constants as each other. Thus, the observed splitting spacings of the isotopic derivatives are well coincided with the expected ones which can be derived by assuming that $A_H=A_{Nring}=A_{Nsub}=5.70$ gauss, as illustrated in Fig. 3. Although these three coupling constants were thus shown to be very close to each other, it is evident that they are not exactly same since a considerable dissimilarity is seen in the spectra between compounds II and III. In order to determine which proton is most contributed to the spin-coupling mentioned above, four deuterated derivatives, V, VI, VII, and VIII,[12-14] were examined, electron spin resonance spectra being shown in Fig. 4. 3,5,6,7,8-Pentadeuterio derivative (VIII) gave quintet signal, indicating that one of these protons is

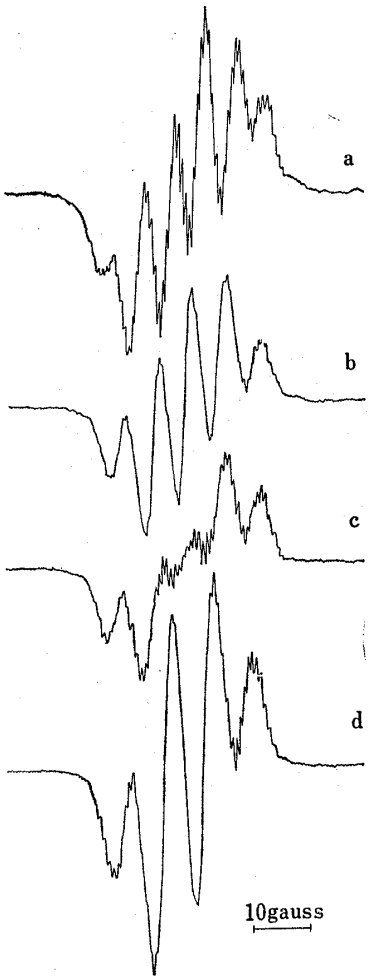

Fig. 2. Electron Spin Resonance Spectra of the Free Radicals produced from (a) O,O'-Diacetyl-4-hydroxyaminoquinoline 1-Oxide (I), (b) Its Hydroxyamino-^{15}N- (II), (c) Quinoline-^{15}N- (III), and (d) Di-^{15}N- (IV) Derivatives in Dioxane. Modulation Width was 4.0 gauss in All Cases

12) N. Kataoka, A. Imamura, Y. Kawazoe, G. Chihara, and C. Nagata, *Chem. Pharm. Bull.* (Tokyo), **14**, 897 (1966).
13) Y. Kawazoe, M. Ohnishi, and N. Kataoka, *Chem. Pharm. Bull.* (Tokyo), **13**, 396 (1965).
14) Y. Kawazoe and M. Ohnishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 826 (1967) and the unpublished data from this laboratory.

Compound	N_{ring}	$N_{sub.}$	Observed[a]	Theoretical[b]
I	^{14}N	^{14}N	⊢—28.5 gauss—⊣	⊢—28.5 gauss—⊣
II	^{14}N	^{15}N	⊢—25.0 gauss—⊣	⊢—25.1 gauss—⊣
III	^{15}N	^{14}N	⊢—24.5 gauss—⊣	⊢—25.1 gauss—⊣
IV	^{15}N	^{15}N	⊢21.6 gauss⊣	⊢21.7 gauss⊣

Fig. 3. Schematic Diagrams of the Observed and Theoretical Electron Spin Resonance Spectra of ^{15}N-Labeled O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxides

a) Measured with a modulation width of 4.0 gauss. No account is taken of the relative signal intensity.
b) assuming
$A_{^{14}N_{ring}}$=5.7 gauss (triplet), resulting in $A_{^{15}N_{ring}}$=8.0 gauss (doublet)
$A_{^{14}N_{sub.}}$=5.7 gauss (triplet), resulting in $A_{^{15}N_{sub.}}$=8.0 gauss (doublet)
A_{3-H}=5.7 gauss (doublet)

the one in question. Among them protons 2, 5, and 8 can be clearly excluded from the case since V and VII gave the sixtet signals just as the non-isotopic diacetate. 3-Deuterio derivative (VI), on the other hand, showed a remarkable spectral change on its electron spin resonance spectrum, leading us to a conclusion that the one proton participating in a largest spin

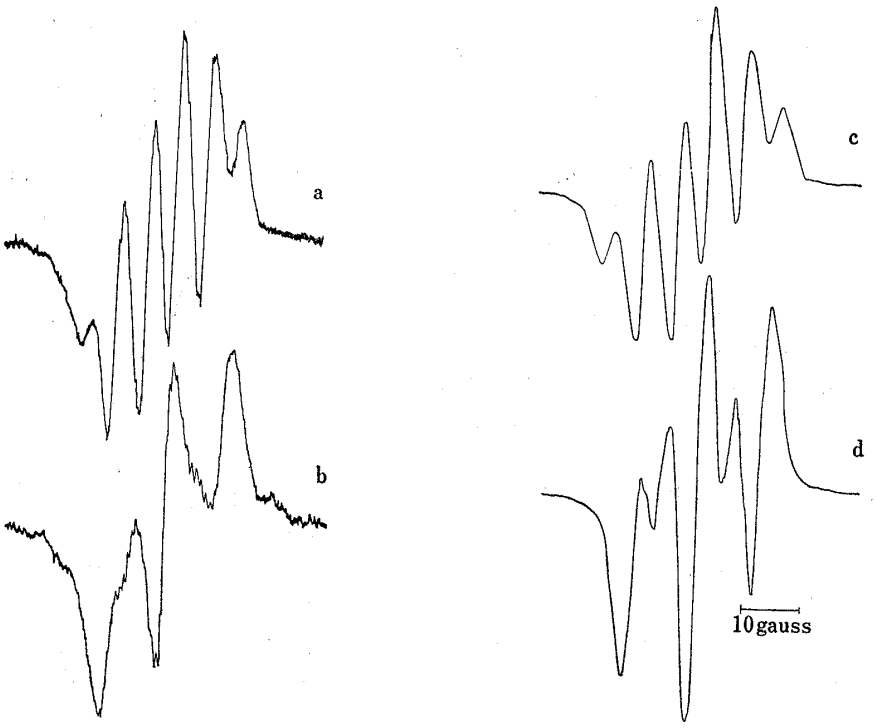

Fig. 4. Electron Spin Resonance Spectra of the Free Radicals produced from (a) 2-Deuterated (V), (b) 3-Deuterated (VI), (c) 5,8-Dideuterated (VII), and (d) 3,5,6,7,8-Pentadeuterated (VIII) O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxides in Dioxane, Modulation Width being 4.0 gauss in All Cases

interaction is probably proton–3. It is further concluded that other five protons in the quinoline ring must be taking place in the spin interaction to much less extent, although they are definitely concerned since forty–eight lines of the original spectrum was more or less deformed in either case of deuterio derivatives, V, VII, or VIII. Another interesting point to be noted is that IX, where six hydrogens of two acetyl groups were replaced by deuterium, gave the completely same spectrum as the non-isotopic derivative even when the spectrum was measured with a modulation of 0.7 gauss or less. This must indicate that the free radical in question had already lost both acetyl groups or, alternatively, that the free radical structure is such that the participation of either acetyl group is neglegibly small in interaction with the odd electron due to its specified electronic structure. It is still open to further investigation at this point.

Now, considering all the electron spin resonance data mentioned above, a question may arise if the free radical studied is identical or not with the one produced by oxidation of 4-hydroxyaminoquinoline 1-oxide at one electron step, which was previously reported.[15–17] Then, the spectra were measured of both compounds which were pre-treated with deuterium oxide under the reaction condition as previously reported. 4-Hydroxyaminoquinoline 1-oxide, where one active hydrogen is included in the hydroxyamino substituent, showed a different spectrum from the original,[16] whereas the diacetate gave the completely same spectrum as the untreated one. The properties of the free radicals from 4-hydroxyaminoquinoline 1-oxide and the diacetate (I) are contrasted in Table II. These facts led us to a conclusion that these two radicals are very similar in the electronic structure but not identical with each other.

Discussion

O,O′-Diacetyl-4-hydroxyaminoquinoline 1-oxide produced a free radical probably starting with the homolytic splitting of an acetoxy radical to result in formation of about one equivalent mole of acetic acid even in anhydrous organic solvent containing no active hydrogen. Deuterium replacement of hydrogens in either acetyl group did not produce any spectral change in the electron spin resonance, whereas all other hydrogens in the quinoline ring were more or less contributed in the spin interaction with the unpaired electron. In particular, both nitrogen nuclei and a proton in 3-position coupled largely with the unpaired electron, compared to the other nuclei concerned. These facts may suggest the reaction schema as shown in

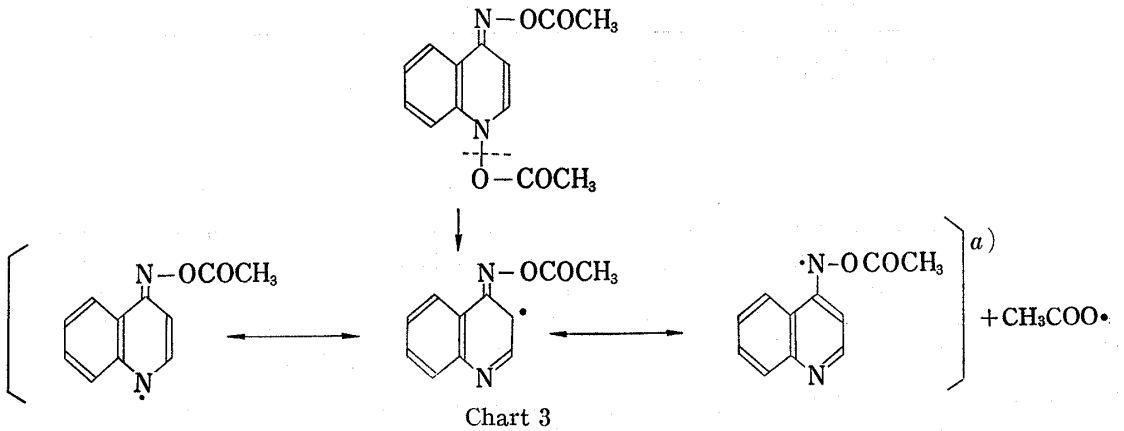

Chart 3

a) These three structures are shown as the canonical formula having main contribution to the spin distribution.

15) C. Nagata, N. Kataoka, A. Imamura, Y. Kawazoe, and G. Chihara, *Gann*, **57**, 323 (1966).
16) N. Kataoka, A. Imamura, Y. Kawazoe, G. Chihara, and C. Nagata, *Bull. Chem. Soc. Japan*, **40**, 62 (1967).
17) N. Kataoka, S. Shibata, A. Imamura, Y. Kawazoe, G. Chihara, and C. Nagata, *Chem. Pharm. Bull.* (Tokyo), **15**, 220 (1967).

Chart 3 when one takes into accout that N-oxide group tends to be readily deoxygenated through free radical reaction process. Further experimental evidences should, however, be needed for definite conclusions of the structure of the free radical and the mechanism of the radical production.

Here, an attention may be called to the correlation of the radical formation with the carcinogenesis of aromatic hydroxylamine derivatives. Preliminary electron spin resonance data indicated that carcinogenic hydroxylamines such as N-hydroxy-2-acetylaminofluorene, N, O-diacetyl-2-hydroxyaminofluorene, and 4-(N-methyl-O-benzoyl)-hydroxyaminoazobenzene gave intense electron spin resonance signals split into a triplet which is due to the spin interaction with the substituent nitrogen nucleus. The free radical formation was observed by warming the dioxane or benzene solutions of these carcinogens at about 70° for a short period of time. It is worth emphasizing that these chemical carcinogens including 4-hydroxyaminoquinoline 1-oxide and its diacetate, which are regarded as the proximate carcinogens, readily produced free radicals, whereas no electron spin resonance signals were observed with the masked forms of the carcinogens, such as 4-nitroquinoline 1-oxide, 2-acetylaminofluorene, and 4-dimethylaminoazobenzene, which are considered to be converted metabolically into the proximate carcinogens for tumor–induction. It is probable, as a result, that the radical formation may be closely related to the tumor–induction, although some non-carcinogenic derivatives such as 4-hydroxyaminoazobenzene and its N,O-diacetate, gave the electron spin resonance signals under a similar reaction condition. Details are now being pursued along this line.

Table II. Comparison of the Property between the Free Radicals produced from O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide and 4-Hydroxyaminoquinoline 1-Oxide

Property	From diacetate	From 4-HAQO[a]
g–Value	2.0048	2.0046
Coupling constant (in dioxane)		
N_{ring}	5.70 gauss	5.9 gauss
N_{sub}	5.70	5.9
3-H	5.70	5.9
2-H	0.7	3.0
5,6,7,8-H's	*ca.* 0.73	1.5 and 0.74
No. of active hydrogen[b]	none	one (A=1.5)

a) 4-hydroxyaminoquinoline 1-oxide
b) Those which are exchangable with active hydrogens in water.

Experimental

Acetyl Migration from O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide to Aniline——In 1.0 ml of pyridine were dissolved 260 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide and 93 mg of aniline and placed in a flask, which was degassed two times. The reaction mixture was kept at 80° for 4 hr. After pyridine was evaporated *in vacuo*, the oily residue was extracted with ether. The extract was chromatographed through an alumina column, eluted with benzene. From the second fraction, acetanilide was obtained. Yield, 26%.

Formation of Acetic Acid and Esters from O,O′-Diacetyl-4-hydroxyaminoquinoline 1-Oxide——In Water: The suspension of 130 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide in 2 ml of H_2O was placed in a tube, degassed two times, and then warmed at 70° for 3 hr. From the colored reaction mixture, volatile fraction was collected by vacuum distillation and diluted with 2 ml of H_2O. Then, the distillate was titrated with 0.1 N NaOH, indicating that 1.7 equivalent mole (30.4 mg) of acetic acid had been liberated.

In Methanol: A solution of 130 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide in 2 ml of absolute methanol was placed in a tube and degassed two times, which was warmed at 70° for 3 hr. The volatile fraction was collected by vacuum distillation and diluted with 2 ml of H_2O. The titration of the distillate

just as the case in water indicated the liberation of 11 mg (0.4 equivalent mole) of acetic acid. To this solution titrated, a known amount of 0.1 N NaOH was added and warmed at 80° for 2 hr. The hydrolysate thus obtained was titrated with 0.1 N HCl. The result indicated that no acetic acid was liberated any more.

In Isopropanol: A solution of 260 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide in 2 ml of absolute isopropanol was placed in a tube and degassed two times, which was warmed at 70° for 3 hr. The same treatment of this reaction mixture just at the case in methanol indicated the liberations of 59.8 mg (1.0 equivalent mole) of acetic acid and of another 24 mg (0.4 equivalent mole) of acetic acid by hydrolysis of isopropylacetate.

In *tert*-Butanol: A solution of 130 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide in absolute *tert*-butanol was placed in a tube and degassed two times, which was warmed 70° for 3 hr. The same treatment of this reaction mixture just as the case in methanol indicated the liberation of 25 .5mg (0.9 equivalent mole) of acetic acid and of another 8.4 mg (0.3 equivalent mole) of acetic acid by hydrolysis of *tert*-butylacetate.

In Dioxane: A solution of 130 mg of O,O′-diacetyl-4-hydroxyaminoquinoline 1-oxide in 2 ml of dioxane dried over Na wire was placed in a tube and degassed two times, which was warmed at 70° for 3 hr. The titration of the distillate from the reaction mixture indicated 25 mg (0.9 equivalent mole) of acetic acid.

Measurements of Electron Spin Resonance Spectra——All the spectra were measured in a quartz tube, using a Japan Electron Optics Laboratory JES-3BX spectrometer with a 100 kc/s modulation. All the spectra were obtained at room temperature.

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