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The Structure of the Free Radical Produced from Carcinogenic 4-Hydroxyaminoquinoline 1-Oxide*

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A potent carcinogen, 4-hydroxyaminoquinoline 1-oxide, was found to produce a stable free radical oxidatively. The structure of the free radical produced in dioxane was studied by analyizng the hyperfine structure of isotope-substituted 4-hydroxyaminoquinoline 1-oxides, and by comparing the values of the spin densities obtained from the coupling constants with those calculated by the unrestricted SCF MO method. It was concluded that the free radical has the same molecular skeleton as the parent compound, but that it loses a hydrogen atom on the nitrogen atom of the NHOH group.

Since Nakahara *et al.*[1-4] found that 4-nitroquinoline 1-oxide (4-NQO) and its several derivatives had a potent carcinogenicity, many studies have been carried out to clarify the mechanism of the carcinogenic action of these compounds. Among the findings, the facts that 4-NQO is converted to 4-aminoquinoline 1-oxide *via* 4-hydroxyaminoquinoline 1-oxide (4-HAQO) *in vivo* as well as *in vitro*,[5-7] and that 4-HAQO also has a carcinogenic activity[8-9] are of especial importance. As 4-aminoquinoline 1-oxide, which is a reductive product of 4-HAQO, was found to be non-carcinogenic, the oxidation-reduction pathway between 4-NQO and 4-HAQO is deemed to be intimately connected with the carcinogenicity, and from the experimental finding that 4-HAQO was more potent in producing a tumor than 4-NQO,[8,9] 4-HAQO is regarded as a more proximate carcinogen than 4-NQO.

The present authors found that 4-HAQO produced stable radicals oxidatively in such organic solvents as dioxan, methanol and acetonitrile. They were formed also in an aqueous alkaline solution to give strong ESR signals. The condition for the free radical production and the possible role of the free radical in the oxidation-reduction process of 4-HAQO were reported in our

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* This paper constitutes Part IV of a series entitled "ESR Studies of 4-Nitroquinoline 1-Oxide and Its Related Compounds." This work has been reported at the Fourth ESR Symposium in Nagoya (Oct., 1965).

[1] W. Nakahara, F. Fukuoka and T. Sugimura, *Gann*, **48**, 129 (1957).

[2] W. Nakahara, F. Fukuoka and S. Sasaki, *ibid.*, **49**, 33 (1958).

[3] W. Nakahara and F. Fukuoka, *ibid.*, **50**, 1 (1959).

[4] W. Nakahara, *Arzneim. Forsch.*, **14**, 842 (1964).

[5] T. Okabayashi and A. Yoshimoto, *Chem. Pharm. Bull. (Tokyo)*, **10**, 122 (1962).

[6] T. Sugimura, K. Okabe and H. Endo, *Gann*, **56**, 489 (1965).

[7] H. Hoshino, F. Fukuoka, K. Okabe and T. Sugimura, *ibid.*, **57**, 71 (1966).

[8] Y. Shirasu and A. Ohta, *ibid.*, **54**, 221 (1963).

[9] H. Endo and F. Kume, *Naturwiss.*, **50**, 525 (1963); *ibid.*, **54**, 443 (1963).

previous paper.[10] The present paper will report the structure of the free radical thus produced in dioxan by analyzing the hyperfine structure of the ESR spectra of 4-HAQO and its isotope-substituted compounds, and by correlating the spin coupling constants with the spin densities calculated by the unrestricted SCF MO method.

Materials and Methods

Syntheses of Isotope-labeled Compounds. The isotope-labeled compounds of 4-HAQO used in this experiment were synthesized by the catalytic hydrogenation of labeled 4-NQO's,[11,12] and purified by recrystallization from 20% hydrochloric acid. Labeled compounds of 4-NQO were synthesized as described in our previous paper.[13]

The Removal of Oxygen. The removal of oxygen, which was necessary for obtaining clear hyperfine structures, was carried out by a usual degassing method using a high-vacuum pump to the extent of 10^{-4} mmHg.

The Measurement of the ESR Spectra. All the ESR spectra were measured in quartz tube in dioxan solutions saturated† with the compounds examined, using a Japan Electron Optics Laboratory JES-3BX spectrometer with a 100 kc/s field modulation. All the measurements were carried out at room temperature after degassing to the extent of 10^{-4} mmHg.

Results and Discussion

In the presence of a small amount of molecular oxygen, 4-HAQO was found to be easily converted to a free radical in dioxan.

When the modulation width is 0.7 gauss, this radical shows a hyperfine structure consisting of 52 lines with a *g*-value of 2.0046 (Fig. 2a).

The Effect of Oxygen. As was described in our previous paper,[10] oxygen is necessary for the free radical formation from 4-HAQO in a dioxan solution. This was confirmed by the following experiment. That is, the dioxane solution suspended with an excess of 4-HAQO was degassed to the extent of 10^{-4} mmHg, and then sealed in a quartz tube. In this condition, signals of the free radical were observed; probably a trace of oxygen dissolved in the solvent was used for the free radical formation. On heating the sample at 80°C, the signal intensity was much increased. Then, the radicals were destroyed by photoirradiation, no signal being observed anymore. Then the signal appeared and increased when the sample was heated again; this procedure was repeated until the trace of oxygen in the solution was consumed completely. In this condition no signal was observed, showing that no radical formation occurs any longer. However, as soon as this solution was exposed to air, the signal appeared instantaneously. This shows that oxygen is necessary for the radical formation and that the free radical is produced *via* the oxidation process.

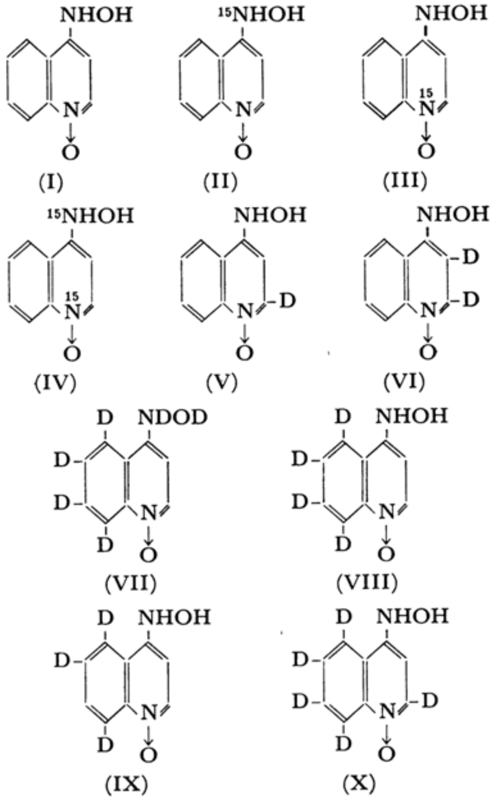

Fig. 1. ^{15}N and D-replaced compounds synthesized for the determination of the structure of the free radical produced from 4-HAQO.

The Determination of the Structure of the Free Radicals by Isotope Replacement. It would be impossible to determine the structure of the free radical produced from 4-HAQO without the data from the appropriate labeled compounds. In fact, isotope-substitution was very effective in elucidating the radical structure in our case. Thus, nine of the ^{15}N and D-replaced compounds listed in Fig. 1 were synthesized for an analysis of the hyperfine structure of the ESR spectra.

Since ^{15}N and D have different spin numbers (I) and magnetogyric ratios (γ) from those of ^{14}N and H respectively, the replacement of ^{14}N by ^{15}N is expected to lead to a significant change in

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[10] C. Nagata, N. Kataoka, A. Imamura, Y. Kawazoe and G. Chihara, *Gann*, **57**, 323 (1966).

[11] E. Ochiai and T. Naito, *Yakugaku Zasshi (Tokyo)*, **64**, 206 (1964).

[12] E. Ochiai, A. Ohta and H. Nomura, *Chem. Pharm. Bull. (Tokyo)*, **5**, 310 (1957).

[13] N. Kataoka, A. Imamura, Y. Kawazoe, G. Chihara and C. Nagata, *ibid.*, **14**, 897 (1966).

† Each saturated solution contains about 0.002 wt% of the compounds.

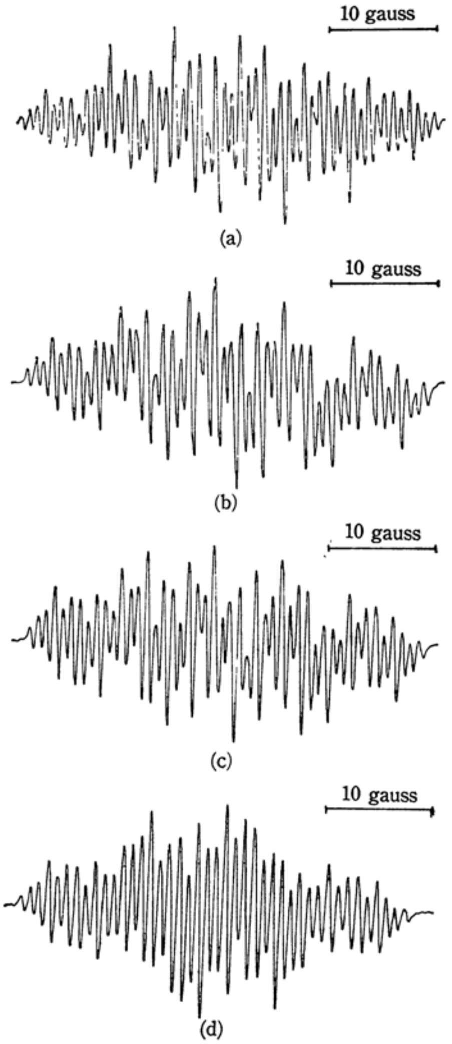


Fig. 2. ESR signals of the free radicals produced from 4-HAQO and its ^{15}N-labeled compounds in dioxan, and modulation width is 0.7 gauss. (a): I, (b): II, (c): III and (d): IV.

the ESR signal pattern (from a triplet to a doublet) and in magnitude of the coupling constant, A ($A(^{15}\mathrm{N})=1.403A(^{14}\mathrm{N})$). The replacement of H by D also causes remarkable spectral changes (from a doublet to a triplet and $A(\mathrm{D})=0.154\,A(\mathrm{H})$).

The ESR Signal of ^{15}N-Labeled Compounds. Either the ring nitrogen (N_{ring}) or the hydroxyamino nitrogen (N_{NHOH}) of 4-HAQO was replaced by ^{15}N; the hyperfine structures of the free radical produced from these compounds are shown in Figs. 2b and c. In comparison with the signal in Fig. 2a, one must notice a remarkable change in the signal pattern. This may reasonably be expected if we consider the difference in coupling constants and spin numbers between ^{15}N and ^{14}N. On the other hand, the signals in Figs. 2b and c are exactly the same in the shape and number of the split lines; this indicates that the unparied electron is equally distributed on the two nitrogens, N_{ring} and N_{NHOH}. The signal of the compound in which both of the nitrogens were replaced by ^{15}N confirms this conclusion (Fig. 2d),

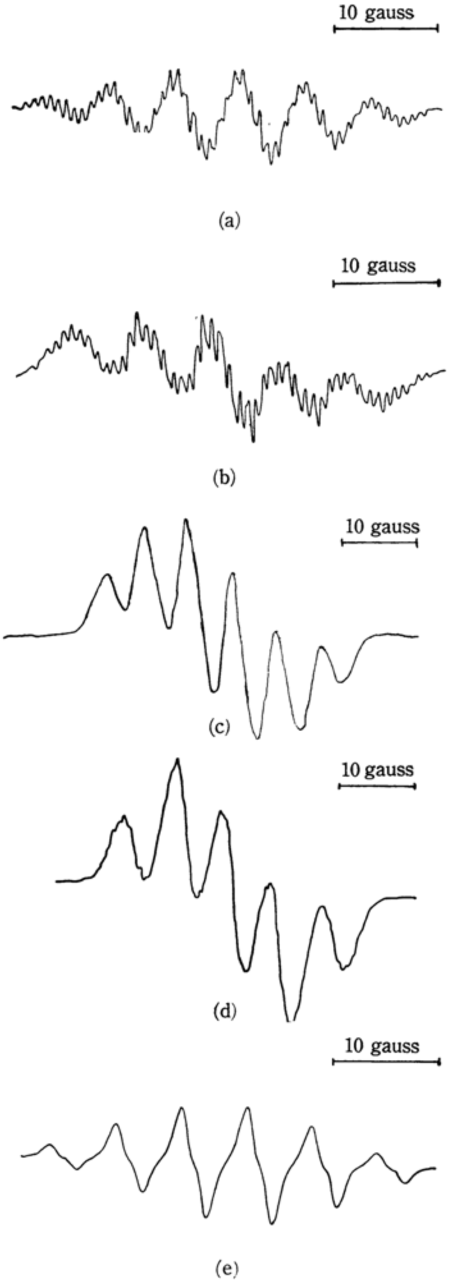


Fig. 3. ESR signals of the free radicals produced from D-replaced 4-HAQO's and the signals necessary for the illustration of h. f. s. of them. Modulation width is 0.7 gauss in the cases of (a), (b) and (e), and 4 gauss in the cases of (c) and (d). (a): V, (b): VI, (c): I, (d): II, and (e): X.

and the coupling constants for the two nitrogens have been found to be exactly equal.

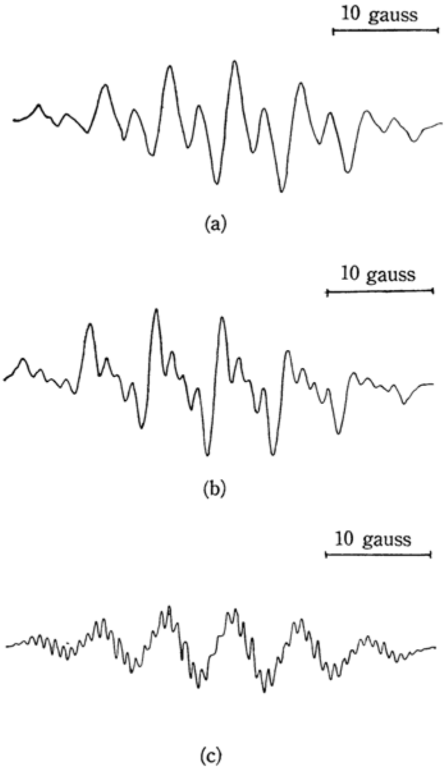
The ESR Signal of D-Replaced Compounds. Figures 3a and b reproduce the spectra of 2-deutero-(V) and 2, 3-deutero-4-HAQO (VI). The former has six main lines in contrast to the five of the latter. This shows that the coupling constant of a proton at the 3 position is so large that the shape of the signals changed greatly. The parent compound, non-labeled 4-HAQO, gave a signal consisting of six broad lines when the measurements were carried out at the modulation width of 4 gauss (Fig. 3c). The compound in which either of the two nitrogens was replaced by ^{15}N gave five broad lines (Fig. 3d) under the same measurement conditions. These data, together with the signals in Fig. 3a and Fig. 3b, show that the proton at the 3 position has nearly the same coupling constant as the two nitrogens. Thus, the six broad lines of the parent compound can reasonably be analyzed if one assumes that the quintetly split lines due to two equivalent nitrogens are further doubly split by the proton at the 3 position. This analysis is supported by the signal of the 2, 5, 6, 7, 8-deuterated compound (Fig. 3e), which consists of six main absorption lines; the coupling constant of the two nitrogens and the proton at the 3 position is obtained as 5.9 gauss from the signals in Figs. 3a and e.

Figure 4a is the spectrum of a hexadeutero compound (VII), where all the proton couplings were extinctured except for those with 2 and 3 protons. Twelve lines are well explained by a further splitting into a doublet of each of the six main lines due to the 2 proton, the coupling constant being found as 2.3 gauss.

Figure 4b was obtained from the 5, 6, 7, 8-deuterated compound (VIII), in which 24 lines are observed. These lines can be analyzed as a further doublet-splitting of the 12 lines in Fig. 4a, a splitting due to one of the two protons of the NHOH group. It may be concluded, from this fact, that the NHOH group has only one hydrogen atom, having lost one of the original two. The coupling constant of the remaining hydrogen atom of the NHOH group was found from Fig. 4b to be 1.3 gauss.

The coupling constant of the 7 proton is estimated from the signal of the 5, 6, 8-deuterated compound IX (Fig. 4c). The value, about a half of the coupling constant of a proton of the NHOH group, was determined to be 0.74 gauss.

From the above experimental results, the following two sets may be adopted as appropriate coupling constants. In the reconstruction of the spectrum, the coupling constants of the 5, 6, and 8 positions, whose coupling constants could not be estimated from the observed signals, were appropriately so evaluated that the final 52, 47, and 42 lines could be obtained for ^{14}N- and ^{15}N-containing compounds I, II and III, and IV respectively. Here, the two sets of coupling constants shown in Table 1 were chosen. Assuming the values of the coupling constants in Set I, 52 lines of the hyperfine structure of the free radical are reconstructed as in Fig. 5.



(a)

(b)

(c)

Fig. 4. ESR signals of the free radicals produced from D-replaced compounds of 4-HAQO in dioxane. Modulation width is 0.7 gauss. (a): VII, (b): VIII, and (c): IX.

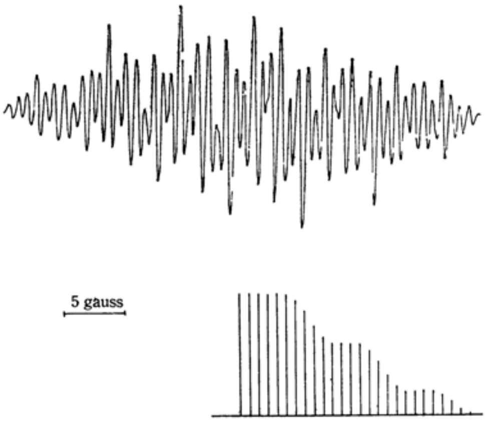


Fig. 5. Hyperfine structure of the free radical produced from 4-HAQO in dioxan and reconstruction based on the coupling constants in Table 1.

In order to determine the structure of the free radical, and also to determine which pair in Table 1 is more appropriate, the coupling constants experimentally obtained were compared with the spin densities calculated by the unrestricted SCF MO method.[14]

TABLE 1. THE COUPLING CONSTANTS OF NITROGEN AND PROTONS AS DETERMINED FROM THE HYPERFINE STRUCTURES OF THE PARENT AND ISOTOPE LABELED 4-HAQO'S

I		II	
A_N	5.9	A_N	5.9
$A_{proton\text{-}3}$	5.9	$A_{proton\text{-}3}$	5.9
$A_{proton\text{-}2}$	3.0	$A_{proton\text{-}2}$	3.0
$A_{proton\text{-}5}$	1.5	$A_{proton\text{-}5,8}$	1.5
$A_{proton\ at\ subst.}$*	1.5	$A_{proton\ at\ subst.}$*	1.5
$A_{proton\text{-}6,7,8}$	0.74	$A_{proton\text{-}6,7}$	0.74

* Proton on the substituent at the 4 position.

The three structures shown in Fig. 6 were adopted for that of the free radical for the following reasons: 1) The free radical produced from 4-HAQO is found to have the almost same molecular skeleton as the parent molecule; the isotope substitution of a proton at any position in the molecule affected the signal pattern. 2) From Fig. 4b, it may be concluded that only one of the substituent hydrogen atoms, H_{NHOH}, exists in the free radical. 3) As has been stated above, oxygen is necessary for the formation of this radical; this suggests that the free radical is formed *via* oxidative dehydrogenation.

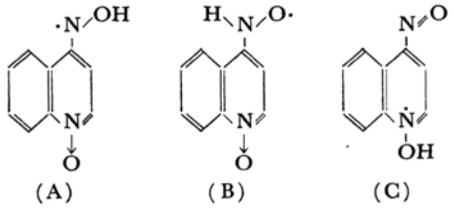


Fig. 6. Three possible structures of the free radical produced from 4-HAQO.

Possible structures in accordance with these facts are restricted to the three types shown in Fig. 6.

The SCF MO calculation of these structures was carried out; the calculated spin densities are shown in Fig. 7. The details of the calculations are given in the Appendix, while the coupling constants calculated from these spin densities are given in Table 2.

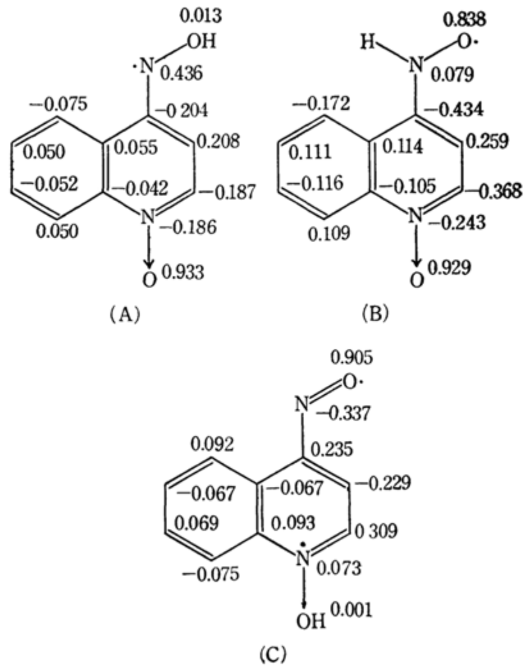


Fig. 7. Spin densities of possible structures calculated by unrestricted SCF MO method.

TABLE 2. THE COUPLING CONSTANTS FOR NITROGENS AND PROTONS IN STRUCTURS A, B, AND C IN FIG. 6, AS CALCULATED FROM EQS. (1) AND (2)

Atoms	Structure A	Structure B	Structure C
N_{ring}	4.71	6.15	1.85
N_{NHOH}	11.03	2.00	8.53
Proton-2	4.21	8.28	6.95
Proton-3	4.68	5.83	5.15
Proton-5	1.69	3.87	2.07
Proton-6	1.13	2.50	1.51
Proton-7	1.17	2.61	1.55
Proton-8	1.13	2.45	1.69

The coupling constants for protons were estimated from the spin densities on carbon atoms to which the hydrogen atoms were attached according to the following equation:

$$A_H = Q\rho_C \quad (1)$$

where A_H denotes the coupling constant due to a proton and Q is the constant which is connected with the σ-π interaction.

Usually, Q is taken to be 22.5 gauss. With regard to the nitrogen atom, the relation between the coupling constant and the spin density may be represented as follows:

$$A_N = Q\rho_N \quad (2)$$

where Q was taken to be 25.3 gauss by Carrington and Santos-Veiga, who considered the effect of the polarization by an adjacent atom to be

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[14] J. A. Pople and R. K. Nesbet, *J. Chem. Phys.*, **22**, 571 (1954).

negligible.[15]

In comparing the coupling constants calculated from the spin densities (Table 2) with those in Table 1, the present authors adopted the structure A as the most probable one for the free radical produced from 4-HAQO. Thus, the observed coupling constant of the 3 proton is larger than that of the 2 proton; only the coupling constants in structure A conform to this fact, although the difference between $A_{\text{proton-2}}$ and $A_{\text{proton-3}}$ is very small in comparison with the experimental difference. Furthermore, from a comparison of the relative magnitude of the coupling constant of the 5 proton with those of the other protons, structure A seems to be the best among the three structures.

Experiments show that the coupling constants of both nitrogen atoms and the 3 proton are nearly equal. This fact is difficult to explain on the basis of the calculated coupling constants; this is probably to be ascribed to the incompleteness of the approximation adopted.

To determine which set in Table 1 is more appropriate is difficult, but from a comparison of the coupling constants in Tables 1 and 2, set I seems to be more adequate.

As has been stated above, structure A was regarded as the most probable one, but the possiblility of the other structures, B and C, can not be completely denied. In order to make a further study of this point, an experiment not using the ESR method should be carried out. The preliminary data of the infrared spectrum method obtained in this Institute support the conclusion obtained from the ESR method.

It is worth noting that the spin concentration found by ESR measurements was one-tenth or more of the original concentration of 4-HQAO. This shows that this radical formation reaction proceeded quantitatively.

We reported in a previous paper[10] that 4-HAQO is oxidized to a free radical in alkaline water. Although the structure of that radical has not yet been determined, that radical seems to be in the same oxidative stage of 4-HAQO as the one obtained in dioxane. This was strongly suggested from their similar chemical behavior.[10]

Further studies of the radical in water are now in progress in connection with its biological role in carcinogenicity.

The authors wish to thank Dr. Waro Nakahara, Director of this Institute, for his continued interest and encouragement in this work. Thanks are also due to Miss Sachiko Shibata of Kitazato University for her technical assistance. This work has been supported in part by a grant from the Ministry of Education to the Carcinogenesis.

Appendix

For the π electron system, the Hamiltonian is written as follows;

$$\boldsymbol{H} = \sum_{i} \boldsymbol{H}_{i\ \text{core}} + \sum_{i>j} \frac{e^2}{r_{ij}} \tag{A-1}$$

and the molecular orbitals are represented by a linear combination of atomic orbitals:

$$\phi_i = \sum_{r} C_{ir} \chi_r \tag{A-2}$$

where χ_r denotes the atomic orbital at the r site and where C_{ir} is the coefficient of the atomic orbital, χ_r, in the ith molecular orbital. In the unrestricted LCAO SCF method, the following equations are given for α and β spin electrons respectively:

$$\sum_{s} F^{\alpha}_{rs} C^{\alpha}_{is} = E^{\alpha}_{i} C^{\alpha}_{is} \tag{A-3}$$

$$\sum_{s} F^{\beta}_{rs} C^{\beta}_{is} = E^{\beta}_{i} C^{\beta}_{is} \tag{A-3$'$}$$

where:

$$\left.\begin{aligned} F^{\alpha}_{rr} &= I_{rr} + \sum_{s} (P^{\alpha}_{ss} + P^{\beta}_{ss})(rr|ss) - P^{\alpha}_{ss}(rr|rr) \\ F^{\alpha}_{rs} &= I_{rs} - P^{\alpha}_{rs}(rr|ss) \end{aligned}\right\} \tag{A-4}$$

$$\left.\begin{aligned} F^{\beta}_{rr} &= I_{rr} + \sum_{s} (P^{\alpha}_{ss} + P^{\beta}_{ss})(rr|ss) - P^{\beta}_{rr}(rr|rr) \\ F^{\beta}_{rs} &= I_{rs} - P^{\beta}_{rs}(rr|ss) \end{aligned}\right\} \tag{A-4$'$}$$

and in which:

$$P^{\alpha}_{rs} = \sum_{\text{occupied MO's}} C^{\alpha}_{ir} C^{\alpha}_{is} \tag{A-5}$$

$$P^{\beta}_{rs} = \sum_{\text{occupied MO's}} C^{\beta}_{ir} C^{\beta}_{is} \tag{A-5$'$}$$

I_{rr} is the core parameter which corresponds to the energy of the electron at the r site, and I_{rs} is the parameter which was often estimated from the observed UV spectra. Further, $(rr|ss)$ represents the Coulomb repulsion integral between electrons that belong to the χ_r and χ_s atomic orbitals. In these formulas, the differential overlap and penetration integrals are neglected.

The integrals involved in the formula are estimated semiempirically. I_{rr} is represented as Eq. (A-6), neglecting the penetration integrals.

$$I_{rr} = -I_r - \sum_{\substack{s \\ (s \neq r)}} n_s (rr|ss) \tag{A-6}$$

where I_r is the ionization potential of the valence electron on the r atom and where n_s denotes the number of electrons that the s atom donates to the system. I_{rs} was assumed to be proportional to the overlap integral of the χ_r and χ_s atomic orbitals:

$$I_{rs} = l S_{rs} \tag{A-7}$$

where l was tentatively estimated to be -12.65 using the estimated values for I_{rs} in many other molecules.[16]

One set of center Coulomb repulsion integrals was

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[15] A. Carrington and J. dos. Santos-Veiga, *Mol. Phys.*, **5**, 21 (1962).

[16] C. Nagata, A. Imamura, Y. Tagashira and M. Kodama, This Bulletin, **38**, 1638 (1965).

estimated using the Pariser-Parr approximation:[17]

$$(rr|rr)=I_r - A_r \tag{A-8}$$

where A_r is the electron affinity of the valence electron on the r site. In evaluating two center integrals, Mataga-Nishimoto approximation[18] was adopted; that is, $(rr|ss)$ was calculated using the a_{rs} parameter as follows:

$$(rr|ss) = \frac{e^2}{R_{rs} + a_{rs}} \tag{A-9}$$

where R_{rs} is the distance from the r atom to s. When the r and s atoms are of the same species, a_{rs} becomes equal to the value giving the following equation:

$$\frac{e^2}{a_{rr}} = \lim_{R_{rs}\to 0} \frac{e^2}{R_{rs} + a_{rs}} (rr|rr) \tag{A-10}$$

When the r and s atoms belong to different species, a_{rs} was assumed to be equal to the harmonic mean of a_{rr} and a_{ss}. Equations (A-3) and (A-3′) were solved using a chosen set of coefficients, C^{α}_{ir}, C^{β}_{ir}, and Eqs. (A-4) and (A-4′) provide new values for F_{rr} and F_{rs}. The substitution of these values into the secular equation gives a new set of coefficients; these iterative procedures are continued m times until the self-consistency is gained between a set of coefficients, C^{α}_{ir} and C^{β}_{ir}, obtained in $(m-1)$th and mth iteration.

In order to carry out SCF calculations for structures A, B and C, all the C–C bond distances were assumed to be 1.39 Å; the C–N bond distance, 1.35 Å, and the N–O bond distance, 1.15 Å; all the orbitals were tentatively considered to be in sp^2 hybridization, and all structures are supposed to be coplanar, making all the C–C–C and C–N–O bond angles 120°. The ionization potential and the electron affinity for valence electrons were obtained from the table of Hinze and Jaffe.[19] The overlap integral, S_{rs}, which is necessary for the calculation of the I_{rs} integral, was obtained using Slater orbitals.

The values of the one-center Coulomb integral and the ionization potentials of the valence electrons are listed in the table. The spin density at the r atom, ρ_r, is given by Eq. (A-11):

$$\rho_r = P^{\alpha}_{rr} - P^{\beta}_{rr} \tag{A-11}$$

We prepared a program for the electronic computer using the Jacobi method. Convergence was considered to be achieved when the difference between the energy levels of the adjacent cycles of iteration was less than 10^{-3} eV. The calculation was carried out on the NEAC 2101 electronic computer of our institute; the total numbers of iterations were 41, 36 and 45 cycles for the structures A, B and C, respectively.

TABLE I. THE VALUES OF THE IONIZATION POTENTIAL AND OF THE ONE-CENTER COULOMB REPULSION INTEGRAL FOR THE VALENCE ELECTRON

$I_{\dot{C}}$[a]	−11.16 eV	$(\dot{C}\dot{C} \mid \dot{C}\dot{C})$[a]	11.13 eV
$I_{\dot{N}}$	−14.12 eV	$(\dot{N}\dot{N} \mid \dot{N}\dot{N})$	12.34 eV
$I_{\ddot{N}}$	−28.53 eV	$(\ddot{N}\ddot{N} \mid \ddot{N}\ddot{N})$	16.57 eV
$I_{\dot{O}}$	−17.28 eV	$(\dot{O}\dot{O} \mid \dot{O}\dot{O})$	15.27 eV
$I_{\ddot{O}}$	−34.75 eV	$(\ddot{O}\ddot{O} \mid \ddot{O}\ddot{O})$	21.18 eV
$I_{N(\to O)}$[b]	−21.02 eV	(NN | NN)	12.89 eV
$I_{O(\leftarrow N)}$	− 9.56 eV	(OO | OO)	14.68 eV

a) $\dot{N}$ means the nitrogen atom which donates one electron to the system, and $\ddot{N}$ that which donates two electrons to the system. For oxygen and carbon atom, a similar notation is used.

b) The integrals concerning the *N*-oxide group are estimated assuming that the net charge on nitrogen and oxygen are +0.5 and −0.5 respectively.

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[17] R. Pariser and R. G. Parr, *J. Chem. Phys.*, **21**, 466, 767 (1953).

[18] N. Mataga and K. Nishimoto, *Z. Physik. Chem.*, **13**, 140 (1957).

[19] J. Hinze and H. H. Jaffe, *J. Am. Chem. Soc.*, **84**, 540 (1962).