An Investigation of the Three Oxidation Forms of Lumiflavin

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Ab initio calculations with the 3-21G basis set have been performed on the three oxidation forms of lumiflavin, and semiempirical MINDO/3 calculations have also been performed on these systems. The molecular geometries have been obtained and are in good agreement with available experimental data. The electronic distributions for the three oxidation forms have been studied, their differences providing some insight into the main chemical changes that occur to lumiflavin when involved in electron-transfer processes. The spin distribution for the radical form has also been calculated and compared to EPR results. Finally, calculations on the 2+ and 2− charged species for the oxidized and reduced forms have been performed to gain some novel insights into the possible aromatic character of these two compounds.

Flavin is the generic name for the 7,8-dimethyl-10-alkyl isoalloxazine (isoalloxazine = 10-substituted 2,3,4,10-tetrahydrobenzo[g]pteridine-2,4-dione) moiety of flavin nucleotides. These nucleotides are cofactors of many flavoproteins that play an important role in biological redox reactions.[1,2] For example, two common cofactors are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The electronic structure of the flavin together with the interactions with the protein environment are the main factors that determine the flavin reactivity, and in particular the redox potential of flavoproteins.[3,4,5] *In vitro* the simpler isoalloxazines, especially lumiflavin (7,8,10-trimethylisoalloxazine), exhibit much of the redox and chemical properties of the cofactors, provided a 10-alkyl substituent is present.[1,2,6,7] Therefore a detailed knowledge of the molecular structure of the isoalloxazine ring is of great importance for the understanding of the biological functions of flavoproteins. To this end, this work is interested in the three neutral forms of lumiflavin (Fig. 1).

Many experimental studies have been undertaken for the elucidation of the isoalloxazine molecular structure. X-Ray diffraction studies on substituted isoalloxazines[8-10] have shown that the oxidized form has planar geometry. However, NMR data suggests[11] that N(10) (see Fig. 2 for atomic numbering) does not lie in the molecular plane in aprotic solvents. For the reduced form, crystal structure determinations[12,13] show that the entire isoalloxazine system consists of two almost planar halves folded about the N(5)–N(10) axis (Fig. 3). Spectroscopic data of reduced flavins have also been interpreted in terms of a bent structure.[14] The barrier for the ring inversion is estimated to be less than 5 kcal mol⁻¹ by a NMR study.[15] The molecular structure of the radical form is less well known. Its instability has not permitted crystal structure determination. EPR and ENDOR spectroscopy studies on flavins and flavoproteins suggest that the isoalloxazine ring for this form may be fairly coplanar.[16,17]

The elucidation of the molecular structure of simpler isoalloxazines has been the goal of many theoretical investigations. Most of this work was performed using semiempirical methods, including extended Hückel MO,[18] CNDO/2,[18,19] PPP,[19,21] PRDDO,[22] and MINDO/3.[23,26] Of these, only Hall *et al.*[23,24] undertook complete geometry optimizations. In comparison only two *ab initio* works have been reported; a study on the
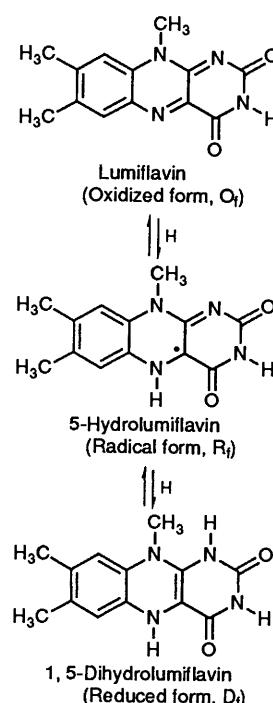
Lumiflavin
(Oxidized form, O_f)

5-Hydrolumiflavin
(Radical form, R_f)

1, 5-Dihydrolumiflavin
(Reduced form, D_f)

Fig. 1 The three oxidation forms of lumiflavin

molecular structure of 10*H*-isoalloxazine and its reduced form (1,5-dihydroisoalloxazine),[27] and a separate study on its radical form.[28] However, no geometry optimizations were done in both works.

Discussion about the aromaticity of the isoalloxazine ring in its oxidized and reduced forms has appeared in the literature. However, there is much confusion surrounding this topic. For example, it has been stated that the oxidized form should be non aromatic[23,29] in contrast to what has usually been considered to be the case.[8,15,30,31] Similarly, the reduced form was referred to as anti-aromatic by Tauscher *et al.*[30] but more recently Hall *et al.* have stated that this is not so.[24,30]

To date, the level of theory applied in the theoretical studies may not be accurate enough to obtain an adequate description of the molecular structure of the isoalloxazine ring. For this reason we have performed *ab initio* SCF calculations with complete geometry optimizations to study the three oxidation forms of lumiflavin. Due to the large size of these systems, the
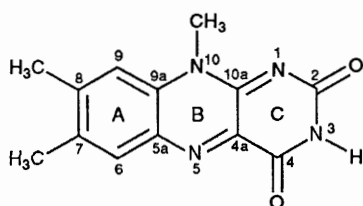
Fig. 2 Atomic numbering for the isoalloxazine ring

calculations would be expensive or impossible on most machines with conventional disc-based codes. Consequently we have used the Direct SCF method[32] as coded at Cambridge.[33] This work mainly addresses the molecular geometry and charge distributions of these systems. Finally, it is also intended to gain some insight into the possible aromatic character of the oxidized and reduced forms. Semiempirical calculations using the MINDO/3 method have also been performed to obtain some additional information on these systems.

Method

Full geometry optimisations at the MINDO/3[34-36] level were performed on the three oxidation species of lumiflavin. The optimized conformations are named as follows: O_f for the oxidized form, R_f for the reduced one, and D_f for the radical form. The subscript f indicates that the structure is obtained from full geometry optimization. For the radical species both UHF and AUHF calculations were performed.

The energetics of ring folding about the N(5)–N(10) axis were studied by performing several calculations with geometric constraints. The predicted MINDO/3 minimum energy structure for the oxidized form (O_f) is essentially planar and so two non-

planar calculations were also done. In these we fixed all the dihedral angles for the ring atoms to be 180° or 0° except C(5a)–N(5)–C(4a)–C(4) and C(9a)–C(5a)–N(5)–C(4a) (see Fig. 2 for atomic numbering); all other parameters were optimized. For the folded structure O_{c1}, these torsion angles were fixed at 170° and 10°, respectively, and in the other (O_{c2}) at 160° and 20°. The subscript c indicates that geometric constraints were established in the calculation. For both the reduced and radical species the minimum energy structure is bent and thus one extra calculation was performed for both species constraining all the atoms other than hydrogen to be in a plane. These conformations will be referred to as R_c and D_c. All semiempirical calculations were performed using the VAMP suite of programs.[37]

Subsequently, we performed *ab initio* calculations using the 3-21G basis set.[38] Full geometry optimizations were undertaken to get the O_f, R_f and D_f conformations, using the corresponding MINDO/3 optimized structures as starting points. Computations with geometric constraints analogous to those described above were also done. It is known that geometries around three-covalent nitrogen atoms calculated with the 3-21G basis set tend to be more planar than the experimental geometries.[39,40] Despite this, our results show that the geometries obtained are still quite reasonable. Similarly, 3-21G calculations underestimate barriers to inversion at three-covalent nitrogen atoms. For this reason, single point calculations using the 6-31G* basis set[41] were performed on the 3-21G optimized geometries of R_f and R_c. With these calculations we expect to get a better estimate for the barrier of the ring inversion for the reduced form of lumiflavin. As in the MINDO/3 calculations, the UHF and AUHF[42,43] methods were used for the computations on the radical form.

Results and Discussion

Ring Folding and Molecular Shapes.—A summary of the 3-21G energies is presented in Table 1. Table 2 shows the main dihedral angles for the O_f, R_f and D_f conformations. In all 3-21G geometry optimizations the largest component of the gradient is less than 7.4×10^{-5} Hartree Bohr^{-1}. The atom numbering of the isoalloxazine ring is indicated in Fig. 2.

Oxidized form. The 3-21G calculations give an essentially planar structure for the most stable conformation of the oxidized form, the largest deviations being *ca.* 2°, occurring at the N(10) atom and the attached methyl carbon atom (see Table 2). The MINDO/3 calculations carried out by Hall *et al.*[23] also yielded small deviations in lumiflavin and other substituted isoalloxazines. Using ^{13}C and ^{15}N NMR techniques Moonen *et al.*[11] have performed a study of several chemically substituted flavins which suggests that the N(10) atom is out of plane in apolar solvents but more in plane in polar solvents. Intuitively one would expect the computed gas phase structure to be closer to the structure in an apolar, rather than polar, environment. If so, the theoretical results are not in agreement with the

Table 1 Relative energies (E_r/kcal mol^{-1}), electronic energies (E_e/Hartree) and nuclear repulsion energies (E_N/Hartree) for the neutral structures studied by the DSCF method with the 3-21G basis set[a]

Structures	E_r	E_e	E_N
Oxidized			
O_f	0.00	−2245.926 48	1383.930 91
O_{c1}	1.95	−2246.583 07	1385.590 60
O_{c2}	8.29	−2246.635 01	1385.652 64
Reduced			
R_f	0.00	−2286.876 62	1423.703 62
	(0.00)	(−2291.708 87)	
R_c	2.40	−2283.511 98	1420.342 81
	(4.68)	(−2284.340 60)	
Radical (R_f)			
UHF		−2263.826 87	1401.239 42
AUHF		−2266.287 17	1403.706 85

[a] Values in parentheses refer to the 6-31G*//3-21G calculations.

Table 2 Significative dihedral angles[a] (in degrees) obtained for the O_f, R_f and D_f structures by the 3-21G and MINDO/3 calculations done in this work

Dihedral angles	Oxidized (O_f)		Reduced (R_f)		Radical (D_f)[b]	
	MINDO/3	3-21G	MINDO/3	3-21G	MINDO/3	3-21G
N(1)–C(10a)–N(10)–C(9a)	−174.5	−178.7	153.4	155.8	168.6	177.6
C(10a)–N(10)–C(9a)–C(9)	175.3	178.4	−153.9	−155.5	−168.3	−177.5
C(4)–C(4a)–N(5)–C(5a)	179.0	179.9	−155.3	−157.0	−175.2	−178.2
C(4a)–N(5)–C(5a)–C(6)	−178.1	179.9	160.5	160.2	175.2	178.3
N(1)–C(10a)–N(10)–CH$_3$	5.1	2.3	−30.8	−73.7	−11.4	0.6
N(4)–C(4a)–N(5)–H	—	—	12.6	5.2	2.8	0.7

[a] Positive values correspond to clockwise rotations, and negative ones to anticlockwise rotations. [b] Values obtained by the UHF method.

Table 3 Geometries around the N(10) and N(5) nitrogen atoms for the R$_f$ structure computed in this work

	Planarity defect at[a]		Substituent orientation at[b]	
	N(5)	N(10)	N(5)	N(10)
MINDO/3	P(1.0)	P(0.1)	E(173)	A(151)
3-21G	P(2.6)	NP(16.1)	E(178)	A(111)

[a] In degrees. Defined as $360° - \Sigma$ (angles around the N atom). P = planarity. NP = nonplanarity. [b] In degrees. For N(5) = $\frac{1}{2}$ [C(10a)–C(4a)–N(5)H + C(4a)–C(5a)–N(5)H]. For N(10) = $\frac{1}{2}$ [C(4a)–C(10a)–N(10)–CH_3 + C(5a)–C(9a)–N(10)–CH_3]. The absolute values for the torsion angles were considered. A = axial. E = equatorial.
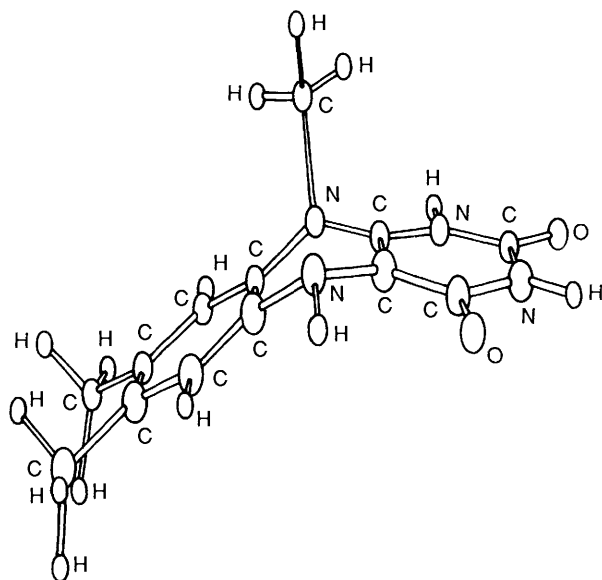
Fig. 3 Most stable conformation of 1,5-dihydrolumiflavin

conclusions of Moonen et al.[11] However, X-ray diffraction studies on 3-methyllumiflavin[9] and 10-methylisoalloxazine[8] predict approximately coplanar structures.

The 3-21G energies of O_{c1} and O_{c2} relative to O_f are 1.95 kcal mol^{-1} and 8.29 kcal mol^{-1}, respectively. These energies can provide an estimate for the energy cost required to change the molecular shape of lumiflavin. A precise description of the overall molecular shape of each conformation would require the tabulation of all bond lengths, bond angles and dihedral angles. However, a single geometric parameter frequently used to describe approximately the molecular shape is the fold angle. (φ). The fold angle is defined as the angle between normals to the least squares planes: N(1)–C(2)–N(3)–C(4)–C(4a)–C(10a)–N(5)–N(10) and C(6)–C(7)–C(8)–C(9)–C(5a)–C(9a)–N(5)–N(10). Using this definition, O_{c1} and O_{c2} have fold angles of ca. 10° and 20°, respectively. Hall et al.[23] report an energy value of 1.1 kcal mol^{-1} to change the fold angle by 10° at the MINDO/3 level. The single point PRDDO calculations on isoalloxazine by Dixon et al.[22] yielded very similar results to ours; a conformation with $\varphi = 10°$ is 2.0 kcal mol^{-1} higher than the planar, and a conformation with $\varphi = 20°$ is 8.5 kcal mol^{-1} higher. All these calculations therefore predict only a slight energy preference for the planar conformation in the isoalloxazine ring.

Reduced form. The minimum energy conformation of the reduced form is folded at the 3-21G level, as shown in Fig. 3. Table 2 lists the main dihedral angles together with those calculated by the MINDO/3 method. The values provided by both methods are quite similar giving a fold angle of ca. 25°. The value determined by the X-ray crystal structure of 9-bromo-1,5-

dihydro-1,3,7,8,10-pentamethylisoalloxazine is 31°,[12] somewhat larger than that computed. Table 3 shows the geometry around the N(5) and N(10) atoms provided by the ab initio and semiempirical methods. Both calculations predict an essentially planar geometry around the N(5) atom and an equatorial orientation for the attached hydrogen. However, the results for the N(10) atom are not in good agreement. Although both methods predict an axial disposition for the methyl group, the MINDO/3 calculations give a more planar geometry around this atom. No experimental data is available to determine which of the two predictions is more correct; however, the methyl group at N(10) in a crystal structure of the related compound 9-bromo-1,3-dimethyl derivative[12] was determined to be axial, in accord with both sets of results.

As outlined previously, 3-21G and single point 6-31G* calculations were performed to give an estimate of the barrier for ring inversion in the reduced form. The results are given in Table 1. If we consider the relative energy of the R$_c$ conformation as an approximation of the inversion barrier, the value predicted at the 3-21G level is 2.4 kcal mol^{-1}. A significant increase is obtained by the 6-13G*//3-21G calculations, the calculated barrier being 4.7 kcal mol^{-1}. The MINDO/3 result of Hall et al.[23] is 1.7 kcal mol^{-1} and that of Dixon et al.[22] (employing the PRDDO method without geometry optimization) is 4.0 kcal mol^{-1}. Moonen et al.[15] have investigated several flavin model compounds in the reduced form. They find that the barrier for the ring inversion is less than 5 kcal mol^{-1}, in good agreement with our computed values. A consequence of this low inversion barrier is that the conformation of the reduced flavin can be easily influenced upon binding to apo-flavoproteins. This may be of importance in the function of the flavin prosthetic group in biological systems.

Radical form. From EPR and ENDOR investigations of flavins and flavoproteins[16,17] it was concluded that in the radical form the isoalloxazine ring must be fairly coplanar. This is completely consistent with our ab initio results for both UHF and AUHF theories. Single point ROHF ab initio calculations with a (7s3p) basis set performed on 1,5-dihydroisoalloxazine[28] predict a folded structure (fold angle ca. 16°) as the most stable conformation with an inversion barrier of 8 kcal mol^{-1}. It seems probable that the lack of geometry optimization in that study may have led to inaccurate conclusions. In our work the largest deviations from planarity of the dihedral angles for the ring atoms are ca. 3° (Table 2).

The predicted MINDO/3 optimized geometry (D$_f$) is clearly bent. From Table 2 it is clear that most of this distortion comes from the N(10) atom. The MINDO/3 energy of a conformation optimized constraining all atoms other than hydrogen to be in a plane (D$_c$ structure) is 0.73 kcal mol^{-1} higher than that of D$_f$. If this value is taken as an approximation to the inversion barrier we find that it is significantly smaller than that for the reduced form. From these results it seems that the radical form does not have a strong preference for a bent conformation.

Bond Lengths and Bond Angles.—The computed bond lengths and bond angles for the most stable conformations of the three oxidation forms of lumiflavin (O$_f$, R$_f$ and D$_f$) are available from the authors on request. For the oxidised and reduced forms the calculated bond lengths were compared to the data obtained from X-ray diffraction studies on an 'ideal' lumiflavin[8] and on 9-bromo-1,5-dihydro-1,3,7,8,10-pentamethylisoalloxazine.[12] In general the 3-21G bond lengths for the oxidized (O$_f$) and reduced (R$_f$) conformations are much closer to the experimental ones than are the MINDO/3 results. The experimental bond angles were obtained by X-ray diffraction on 3-methyllumiflavin[9] and 9-bromo-1,5-dihydro-1,3,7,8,10-pentamethylisoalloxazine.[12] A strict comparison between the calculated and the experimental values cannot be made because the latter

Table 4 Main changes in bond lengths[a] (in Å) and bond angles (in degrees) computed upon the one-electron reduction oxidized ⟶ radical [Δ(D_f − O_f)] and two-electron reduction [Δ(R_f−O_f)] in this work

	Δ(D_f−O_f)		Δ(R_f−O_f)	
	MINDO/3	3-21G	MINDO/3	3-21G
C(4)–C(4a)	−0.022	−0.052	−0.040	−0.039
C(4a)–C(10a)	−0.026	−0.037	−0.102	−0.132
C(4a)–N(5)	0.059	0.072	0.117	0.126
C(9a)–N(10)	0.003	0.029	0.020	0.064
C(10a)–N(1)	0.001	0.008	0.080	0.101
N(1)–C(2)–N(3)	−0.4	0.3	−3.5	−2.7
C(4)–C(4a)–C(10a)	2.1	2.8	5.2	3.5
C(10a)–C(4a)–N(5)	−4.7	−1.9	−6.1	−2.5
C(4a)–N(5)–C(5a)	4.2	1.8	1.7	−2.1
N(5)–C(5a)–C(9a)	−2.9	−1.9	−3.4	−1.8
C(9a)–N(10)–C(10a)	−0.1	−0.1	−3.5	−7.9
N(10)–C(10a)–C(4a)	2.1	2.0	4.4	5.9
C(9a)–N(10)–CH_3	0.6	−8.0	1.2	−4.4
C(10a)–N(10)–CH_3	−0.5	−1.9	2.1	−3.8

[a] The small values are included for comparison.

Table 5 Main changes[a] in net atomic charges computed upon the one-electron reduction oxidized ⟶ radical [Δ(D_f−O_f)], and two-electron reduction [Δ(R_f−O_f)]

	Δ(D_f−O_f)		Δ(R_f−O_f)	
	MINDO/3	3-21G	MINDO/3	3-21G
N(1)	0.00	0.01	0.19	−0.21
C(2)	0.00	−0.01	−0.04	0.12
C(4a)	−0.12	0.06	−0.14	0.01
C(10a)	0.07	0.01	−0.03	−0.11
N(5)	0.17	−0.40	−0.05	−0.42
N(10)	−0.04	0.00	−0.07	0.20
C(5a)	0.01	0.21	0.12	0.23
C(9a)	−0.04	−0.09	−0.07	−0.25
C(6)	−0.05	−0.05	−0.13	−0.09

[a] The small values are included for comparison.

correspond to derivatives of lumiflavin. However, as for the bond lengths, the 3-21G values are closer to the experimental values than are the MINDO/3 ones. For example, in the oxidized form, the 3-21G computed C(10a)–N(1)–C(2) bond angle is ca. 3° closer to the experimental value. For the reduced and radical forms differences of ca. 4–5° are found for the C(10a)–N(1)–C(2), C(2)–N(3)–C(4), C(10a)–C(4a)–N(5) and C(4a)–N(5)–C(5a) bond angles between the 3-21G and MINDO/3 values. It is found that many of the bond lengths computed for the radical form do not fall between the corresponding values for the oxidized and reduced structures.

The main geometric changes resulting upon reduction of lumiflavin are shown in Table 4. Both the MINDO/3 and 3-21G results predict localized bond length changes in the diazadiene fragment [N(1)=C(10a)–C(4a)=N(5)] upon two-electron reduction (oxidized ⟶ reduced). This agrees with the X-ray data.[8,9,12,13] On going from the oxidized to the radical form the largest changes are found in the bonds formed between C(4a) and those atoms connected to it: 3-21G and MINDO/3 predict the C(4a)–N(5) bond to increase by 0.072 and 0.059 Å, respectively. The trends for bond angle changes, i.e. the sign of the changes, predicted by either of the two methods are in general the same. However, the numerical agreement between both results is not that good, as variations of ca. 3° are found. For the 3-21G work the largest change between the reduced (R_f) and oxidized (O_f) form is predicted to be −7.9° occurring in the C(9a)–N(10)–C(10a) bond angle. This change occurs in the radical ⟶ reduced process, and it is mainly

associated with a change of hybridization of the N(10) atom. This is paralleled in the experimental data; the largest bond angle difference between the X-ray values for R_f and O_f does occur in that angle. Of course, one should remember that the X-ray data shown in Table 5 do not correspond to the oxidized and reduced forms of lumiflavin (but of closely related compounds) and thus this agreement should be treated with caution.

Electron Distributions.—The Mulliken net atomic charges were computed for the O_f, R_f and D_f structures. It is well known that this type of analysis is sensitive to the level of theory employed. Thus a direct comparison between those obtained from the MINDO/3 and 3-21G levels cannot be made. However, it is reasonable to expect that trends predicted by one method would be found by the other. For the three structures both 3-21G and MINDO/3 results predict that the ring C (Fig. 2) is by far the most polar fragment of lumiflavin, as expected from the presence of the high polarity carbonyl groups. The 3-21G results also verify the MINDO/3 work of Hall et al.[24] that the O=C–NH–C=O fragment does not experience much change in partial charges upon the two-electron reduction process, whereas the diazadiene part has the greatest variation (see Table 5). From MINDO/3 partial charges, Hall et al.[24] state that the pyrrole-like nitrogen atoms, N(3) and N(10), are rather dissimilar in electronic state and environment as are the two pyridine-like nitrogen atoms, N(1) and N(5). This is somewhat surprising and is not borne out by our ab initio results; the 3-21G partial charges show that N(10) and N(3) are fairly similar to each other as are N(1) and N(5).*

The largest changes in the net partial charges occurring upon the one-electron oxidized ⟶ radical are shown in Table 5. The most significant change is predicted to occur at N(5) by both methods, but they differ on the sign of this change. As expected from the valence structures given in Fig. 1 the partial charges in the rest of lumiflavin are largely unchanged. The one-electron radical ⟶ reduced process results in significant

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* Hall et al. stated in ref. 23 that for the reduced flavins they studied (including 1,5-dihydrolumiflavin) the computed MINDO/3 electronic energy is lower for the bent geometries, and the nuclear repulsion energy is higher. In Table V of that paper there appears a mistake in the values shown for the 153T conformation. In addition, in ref. 29 Hall et al. state the contrary, i.e. that the computed electronic energy for the reduced form is at a minimum for the planar geometry.

Table 6 Atomic spin densities on the heavy atoms, and values of $\langle S^2\rangle$ computed for the radical form (D_f) of lumiflavin

	MINDO/3		3-21G	
	UHF	AUHF	UHF	AUHF
N(1)	0.059	0.024	0.179	0.049
C(2)	−0.004	0.005	−0.113	−0.006
O(12)	0.016	0.007	−0.140	0.027
N(3)	−0.011	0.004	−0.019	0.002
C(4)	−0.027	0.013	−0.209	−0.038
O(14)	0.016	0.030	0.247	0.086
C(4a)	0.462	0.441	0.553	0.516
C(10a)	−0.072	0.004	−0.198	−0.054
N(5)	0.477	0.375	0.379	0.310
N(10)	0.095	0.040	0.070	0.074
C(5a)	−0.471	−0.011	−0.706	−0.045
C(9a)	0.435	0.026	0.668	0.041
C(6)	0.474	0.027	0.750	0.040
C(7)	−0.453	−0.002	−0.748	−0.014
C(8)	0.478	0.019	0.777	0.044
C(9)	−0.444	−0.001	−0.730	−0.019
C(7)–CH₃	0.052	0.000	0.092	0.000
C(8)–CH₃	0.051	0.000	−0.098	−0.003
N(10)–CH₃	−0.041	−0.001	−0.016	−0.005
$\langle S^2\rangle$	1.118	0.750	1.300	0.757
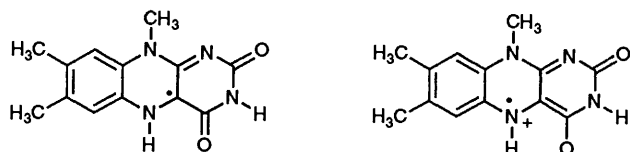
Fig. 4 Two resonance structures for the radical form of lumiflavin

changes in the partial charges at N(1) and N(10). The change in N(1) is obviously related to the change in valence. The change for N(10) is due to its different hybridization as indicated by the N(1)–C(10a)–N(10)–C(9a) fragment which is very much less planar in the reduced form compared to the radical form (see Table 2).

NMR chemical shifts have been compared with partial charges (π or total) obtained from semiempirical calculations in isoalloxazine derivatives.[24,44–46] In most cases little correlation is found. The ¹³C NMR data of Grande et al.[46] on 3-methyl lumiflavin may be used to make a comparison with the computed charges on lumiflavin (oxidized form). If we relate the chemical shift with the population on a given atom then an increase in the total population would be associated with an increase in the chemical shift. The chemical shifts of C(2) and C(4) are the lowest, as predicted by our calculations. The next chemical shift observed is for C(10a) and this is paralleled by our theoretical results. However, several differences appear for the other carbon atoms.

Spin Densities in the Radical Form.—Table 6 lists the atomic spin densities obtained with the 3-21G and MINDO/3 wave-functions. The spin expectation values, $\langle S^2\rangle$, are also given. Comparing the UHF and AUHF values it is apparent that there are some striking differences. In the AUHF method[42,43] the first spin contaminant (the quartet for 5 hydrolumiflavin) is annihilated self consistently, resulting in a $\langle S^2\rangle$ value close to the exact value of $\frac{3}{4}$. As shown by Andrews et al.[47] a variationally optimized, single determinant wavefunction will tend towards the ROHF description as the spin contamination is removed. Thus our AUHF results show a very similar spin density pattern to the ROHF results of Platenkamp et al.[28] where only the C(4a) and N(5) atoms have a significant spin density population. In contrast, the UHF description, by

treating the α and β sets of orbitals separately (and thus introducing some electron correlation), results in a lower variational energy solution but with concomitant spin contamination and significant spin density populations across many atoms in the 5-hydrolumiflavin molecule.

Substituted flavin radicals have been studied by EPR spectroscopy.[2,16,48] Walker et al.[48] concluded that in the flavin radical the spin densities[50] on the C(4a) and N(5) atoms are rather high.* They estimated that the spin density on N(5) was larger than that on the C(4a) atom, contrary to our results. The study of heavily substituted flavins (instead of 5-hydrolumiflavin) in the experimental work may cause some of this discrepancy. In general the AUHF results are in good agreement with what little experimental results there are, and our prediction that the unpaired electron is mostly localized at the C(4a) and N(5) atoms has important implications for the reactivity derived from Frontier Orbital considerations. This prediction also fits with a resonance structure between the two valence-bond representations of the radical shown in Fig. 4. The UHF results are poor because the large spin contamination present (1.30 and 1.19 for 3-12G and MINDO/3, respectively) means that the wavefunction is not a good representation of the real doublet spectroscopic state of the radical.

Aromaticity.—There are several criteria in the literature[49,50] for the classification of aromaticity, thus no universal definition exists. Further, it has been argued that distinguishing compounds in this way is not a useful exercise and that the concept is so vague as to serve no useful purpose. However, there has been so much discussion and confusion in the literature regarding the aromaticity of flavins[8,15,23,29–31] that we considered it interesting to pursue this topic employing a novel type of study.

In this work, we will consider the aromaticity as a special stability due to the conjugation of the π system. Consequently we have undertaken calculations on the 2+ and 2− species for both the oxidized and reduced forms thereby changing the π electron population. Hence any differences in structure between the neutral and charged forms are the result of purely electronic preferences.

The MINDO/3 method predicts that the Highest Occupied Molecular Orbital (HOMO) for the neutral oxidized form is a π orbital. Removal of two electrons from this orbital results in an optimum structure (O^{2+}_f) that is folded. Since the neutral species is planar this suggests that the neutral oxidized form may have some aromatic character. This is further supported by a calculation on the 2+ species constraining it to be planar (all atoms other than hydrogen to be in a plane). This calculation results in a conformation (O^{2+}_c) that is 4.31 kcal mol⁻¹ higher in energy than O^{2+}_f. Hence it seems that there is a reasonable energy preference to go out of plane upon removing two electrons from the oxidized form. This trend is repeated for the 2− species, but the energy differences are smaller. It should be noted that SCF calculations are more difficult to accurately describe diffuse anions, requiring much larger basis sets. This general observation is also probably true for MINDO/3 and as such we will attach more emphasis to the 2+ conclusions.

For the folded reduced form, removal of two electrons gives a planar structure (R^{2+}_f). Calculations on a constrained, folded, structure R^{2+}_c [all dihedral angles for the ring atoms were fixed at 180° or 0° except the C(5a)–N(5)–C(4a)–C(4) and C(9a)–C(5a)–N(5)–C(4a), which were fixed at 160° and 20°, respectively] give an energy of 4.3 kcal mol⁻¹ higher than R^{2+}_f. These

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* The spin densities estimated in ref. 48 refer to spin densities on p_z orbitals. Nevertheless, the comparison with the total atomic spin densities may be made because the contributions from other orbitals to the latter are in general much smaller.

Table 7 Relative energies for different conformations of the same species (E_r/kcal mol^{-1}), electronic energies[a] (E_e/Hartree), and nuclear repulsion energies[a] (E_N/Hartree) computed in this work for oxidized and reduced 2+ and 2− charged species

	Shape	E_r	E_e	E_N
MINDO/3 (geometry optimized calculations)				
O^{2+}$_f$	bent (155°, 171°)[b]	0.00	−19 953.903	16 754.004
O^{2+}$_c$	planar	4.31	−19 901.368	16 701.698
O^{2-}$_f$	bent (163°, 175°)[b]	0.00	−19 932.072	16 713.981
O^{2-}$_c$	planar	0.54	−19 920.850	16 702.783
R^{2+}$_f$	planar[c]	0.00	−20 677.124	17 444.569
R^{2+}$_c$	bent	4.23	−20 695.086	17 462.714
R^{2-}$_f$	planar[c]	0.00	−20 446.456	17 199.542
R^{2-}$_c$	bent	1.76	−20 464.762	17 217.924
3-21G				
O^{2+}$_f$	bent (170°, 178°)			
R^{2+}$_f$	bent (162°, 173°)			

[a] MINDO/3 values in eV, and 3-21G values in Hartree. [b] The first number is the mean value for the torsion angles (absolute values) N(1)–C(10a)–N(10)–C(9a) and C(9)–C(9a)–N(10)–C(10a). The second one is for C(4)–C(4a)–N(5)–C(5a) and C(4a)–N(5)–C(5a)–C(6). [c] The largest deviations from planarity are ca. 2° for torsion angles.

results suggest that the neutral reduced form is not aromatic. All results are given in Table 7.

One of the important deficiencies of the MINDO/3 method is the underestimation of the stability of aromatic compounds.[51] For this reason a few 3-21G calculations were performed to test our conclusions. The computational cost to repeat all the MINDO/3 calculations was considered too prohibitive. Consequently, only the 2+ species were reoptimized. For the oxidized form the 3-21G calculation agrees with MINDO/3 in predicting a bent conformation for the 2+ species—suggesting that the neutral oxidized form may have some aromatic character. For the reduced form the 3-21G calculations predict a bent conformation for both the neutral and 2+ species suggesting that the neutral reduced form is not anti-aromatic.

Our conclusions are not in accord with those of Hall et al.[29] Much of their arguments are based on counting the π electrons and applying the Hückel (4n + 2) rule. From that they suggest that the oxidized form has a tendency for anti-aromaticity, and that the reduced one is more consistent with an aromatic character. It seems that this way of distinguishing the possible aromatic character is not, in general, a straightforward one; assigning and counting electrons is more of an art than a science.

Conclusions

The ab initio calculations carried out in this work on the three oxidation forms of lumiflavin yielded results in good accord with the experimental data. The agreement between the SCF results and those obtained by Hall et al.[23,24] using the MINDO/3 method is reasonably good.

The ab initio calculations predict an essentially planar conformation for the oxidized form, in agreement with the experimental data and the MINDO/3 calculations of Hall et al.[23] MINDO/3 and ab initio calculations on the 2+ species suggest that the isoalloxazine ring may have some aromatic character in this form. The 3-21G results for the reduced form of lumiflavin yield a folded conformation as the most stable one, in accord with the X-ray data on some derivatives and the MINDO/3 results.[23] The structure is predicted to have no aromatic character. The barrier for the ring inversion is predicted to be low and in accord with the upper value of 5 kcal mol^{-1} estimated

by Moonen et al.[15] for several flavins using NMR spectroscopy. The molecular shape predicted for the radical by our ab initio calculations is almost planar, in agreement with EPR and ENDOR investigations on flavins and flavoproteins.[16,17] In contrast, our MINDO/3 results predict a folded conformation as the most stable one, although having a very low barrier for the ring inversion of less than 1 kcal mol^{-1}.

No direct comparison between calculated geometries with experimental ones could be made. Nevertheless, the bond lengths and bond angles computed for the oxidized and reduced forms by the 3-21G calculations are closer to the experimental values of related compounds obtained by X-ray diffraction than are the MINDO/3 ones. The main changes in bond lengths for the two-electron reduction process, computed at the 3-21G level, are associated with the diazadiene fragment. For the one-electron oxidized ⟶ radical reduction our MINDO/3 and 3-21G calculations predict that the major changes in bond lengths are those for the bonds around C(4a); the most important change is that for the C(4a)–N(5) bond. The changes in bond angles computed from each method have in general the same signs but the absolute values are different. The most important change predicted by the 3-21G calculations, being ca. 8° in the C(9a)–N(10)–C(10a) bond angle, occurs in the radical ⟶ reduced process and is supported by X-ray data.

Upon the one- and two-electron reduction the largest changes in the net partial charges, as computed from Mulliken analysis on our ab initio wavefunctions, are predicted to occur in the diazadiene part. However, unlike bond length variations, these changes are not so localized. In fact for the radical ⟶ reduced process the partial charge for N(10) is also predicted to vary significantly. This change is inferred to arise from a difference in hybridization resulting from large variations in the local geometry at N(10).

The spin densities for the radical form of lumiflavin computed by the ab initio and MINDO/3 methods using the UHF approach are meaningless, as the $\langle S^2 \rangle$ expectation values are too highly contaminated. Removal of the first spin contaminant by the AUHF theory leads to $\langle S^2 \rangle$ values much closer to the exact value of 3/4. The spin densities obtained in this way are predicted to be high only at C(4a) and N(5), in agreement with experimental data on substituted flavins.

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