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63. NMR.-Study of Nitrogen Inversion and Conformation of 1,5-Dihydro-isoalloxazines ('Reduced Flavin')¹⁾

Studies in the Flavin Series, XIX. Communication²⁾

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Summary. The pyramidal inversion of the N(5)-centre of several reduced flavins was measured by NMR. The inversion barrier was found to be ~ 10 kcal/mol in acetone solutions and to be independent of the size of the N(5) substituent. An increase of the inversion barrier of ~ 5 kcal/mol was observed in the case where the N(5) substituent could only be in axial position, and an increase of ~ 3.5 kcal/mol was observed for an acyl-like N(5) substituent. In aqueous solution the inversion barrier increases by ~ 3 kcal/mol. The stereochemistry of reduced flavin and its potential relevance in flavin-dependent biological dehydrogenations is discussed.

1. Introduction. – Flavin (vitamin B₂) in its reduced state is derived from either 1,5-dihydro-isoalloxazine (Fig. 1: 1,5-Fl_{red}H₂) or from the 4a,5-dihydro-isomer (4a,5-Fl_{red}H₂). 1,5-Fl_{red}H₂ is formed reversibly and has for many years been taken to be the only possible 'fully reduced' state of flavocoenzymes [1]. We have termed this isomer 'flavohydroquinone' with respect to its thermodynamically reversible formation and its instability to molecular oxygen [2]. The present study is confined to 1,5-Fl_{red}H₂ and its alkyl derivatives. 4a,5-Fl_{red}H₂, which so far is only known as the 4a-alkyl derivative, has been discussed earlier [3].

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²⁾ XVIII. Commun. cf. [6a].

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Flavohydroquinone was first recognized as non-coplanar by virtue of its absorption spectra [4]. Meanwhile, this has been confirmed by crystallography [5]. In the 1,5-Fl_{red}H₂ molecule, the atoms 1, 2, 3, 4, 5, and 10, and 5, 6, 7, 8, 9, and 10 constitute two planes intersecting in the N(5)-N(10) axis with a dihedral angle between 9° and 36°, which depends on the number and size of the N(5) substituents, as shown in Table 1. The present study deals with the dynamic aspects of the stereochemistry (stability) of the so-defined 'butterfly wing' conformations in solution.

1.1. *Ring inversion.* The steric shape of the 1,5-Fl_{red}R₂ (R = H or alkyl) molecule and its possible changes are most efficiently approximated by the 1,4-cyclohexadiene molecule. This simple compound exists in two 'boat' conformations interconverting by ring inversion which involves an energy barrier mainly due to stretching of bond lengths and widening of bond angles (Fig. 1). The ring inversion does not change the configuration of the tetrahedral carbon centres, while it changes axial ligands at these centres into equatorial ones and *vice versa*. This inversion occurs for all practical purpose at two centres at a time, since chair conformations are unstable.

Hence, ring inversion at 1,5-Fl_{red}R₂ means a change of ligand position at N(5) – the 'ligands' being R and a non-bonding electron pair – with retention of chirality

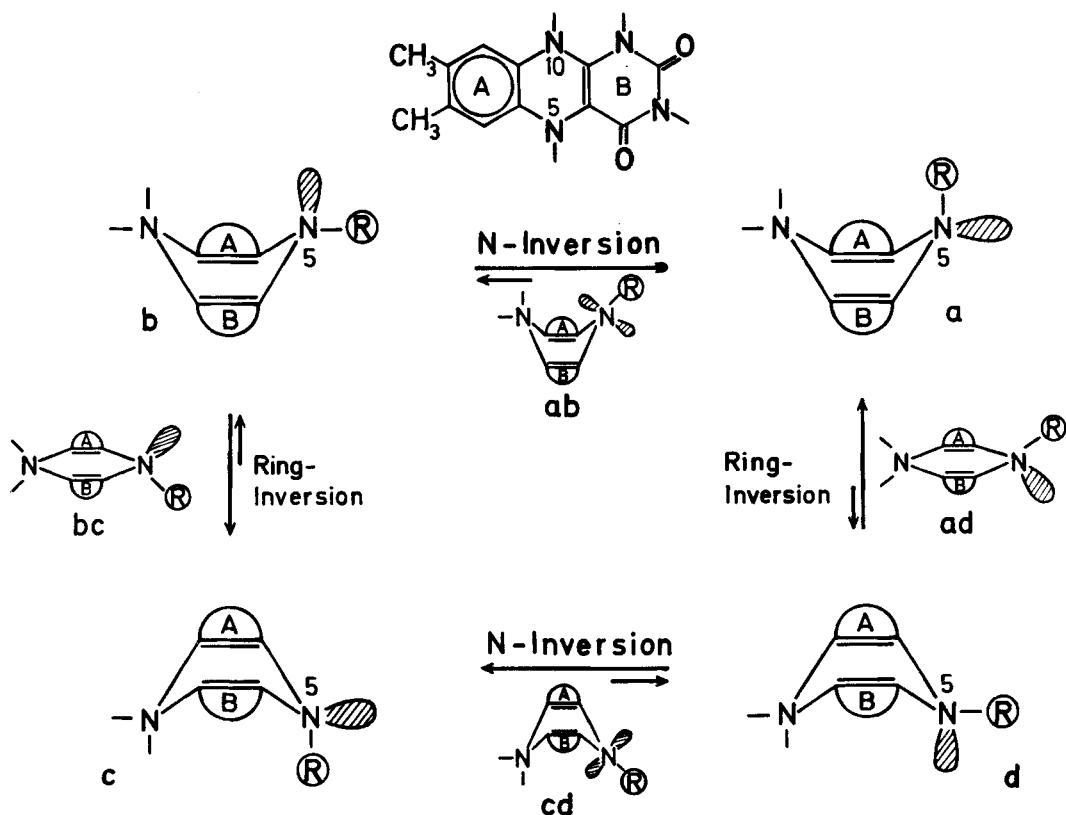


Fig. 1. Configuration changes induced by the different inversion processes in the 1,5-Fl_{red}H₂. A and B representing the aromatic and the pyrimidine side of the flavin nucleus, respectively.

(Fig. 1). This involves, of course, a drastic change in the environment of each ligand and therefore a change of steric hindrance. The more space-filling ligands will always prefer the axial position which minimizes the 'peri' overcrowding effect (Fig. 2). As a consequence, the ring inversion will favour this latter conformer (Fig. 3). The 'para' overcrowding (positions 5 α , 10 α) (Fig. 2) is certainly less pronounced than the 'peri' overcrowding (positions 4 α , 5 α , 6), because the C(5 α)–C(10 α) distance is greater than either C(5 α)–O(4) and C(5 α)–H(6) distances. This is easily seen with the aid of molecular models. Conformers with equatorial R may, therefore, be neglected (b, d in Fig. 1).

1.2. *N*-inversion. In contrast to the ring inversion, the N-inversion involves a change of configuration at the nitrogen centre and proceeds through a planar sp²-

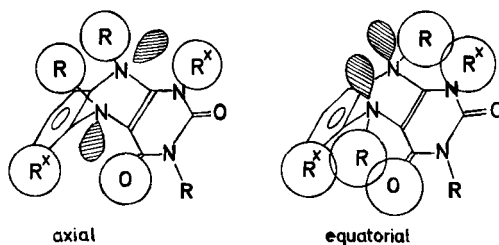


Fig. 2. Overcrowding for the N(5)-ligand R in axial and equatorial position

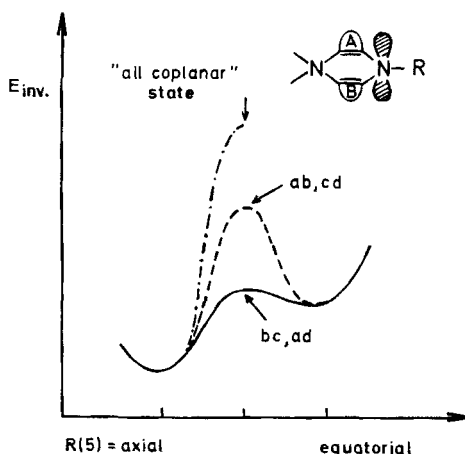


Fig. 3. Energy profile for different inversion processes in the reduced flavin (cf. Fig. 1):

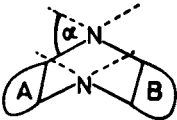
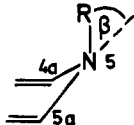
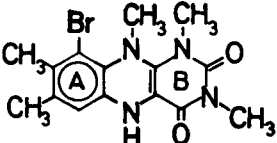
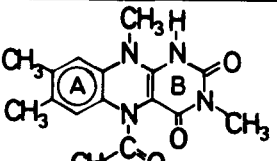
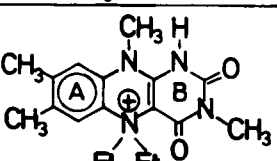
— ring inversion, ---- N-inversion, and - · - · - combined ring and N-inversion

state at N(5) (Fig. 1). However, planarity at N(5) does not necessarily imply coplanarity of the whole molecule. Hence the interconversion of all conformations is possible *without* formation of an 'all-coplanar' intermediate. The dihedral angle of the 'bent' state might, in principle, vary from a maximum of 60° for a fully fledged sp³ tetrahedron down to the measured value of 8.9° [5c] (Table 1) for a system symmetrically substituted at N(5) [6a, b]. The fact that the measured value is this low, must be largely due to the steric influence of the symmetric disubstitution at N(5).

The angle should be greater in the case of asymmetric substitution, as optical spectra demonstrate [6].

The energy barrier of N-inversion (Fig. 3) is given by the energy difference between the 'bent' and the sp^2 configuration (*cf.* Fig. 1a and ab, respectively). This energy difference is the sum of enthalpy and entropy terms. The entropy is mainly determined by the peri-overcrowding which has a maximum in the all-coplanar state but not in the intermediate states ab, ad, bc and cd (Fig. 1). The enthalpy, however, is largely determined by the delocalization of the p_z -electrons in the sp^2 -state at N(5) and N(10), which is 'Hückel-forbidden' in the $Fl_{red}H_2$ nucleus. This nucleus represents an example of an 'antiaromatic' [7] system, the centre (pyrazine) subnucleus containing eight π -electrons. This forbiddenness of the flat state is somewhat lifted by the electron withdrawing 2- and 4-carbonyl groups, and, in addition, in the case

Table 1. Dihedral angles of different reduced flavins in crystalline form

Molecule	angle between A and B planes 	"out of (4a,5,5a) plane" angle 	Ref.
	21°	—	5a
	35.5°	16.2°	5c
	32°	20.6°	5d
	9°	—	5b

$R(5) = H$, by proton delocalization [8]. This phenomenon (also known as tunnel effect) is inversely proportional to the reduced mass of the ligand at the nitrogen centre and is therefore negligible for all ligands heavier than hydrogen [9]. If the peri-overcrowding is small, the 'antiaromaticity' largely governs the actual value of the dihedral angle.

The coincidence of peri-overcrowding – for $R(5) \neq H$ – and 'antiaromaticity' in $1,5\text{-Fl}_{\text{red}}R_2$ renders N-inversion at N(5) slow as compared to ring inversion, while, as will be shown below, inversion remains fast at N(10). This experimental finding can be well understood from the fact that the N(10)-non-bonding pair is much more delocalized than the N(5)-pair into the pyrimidine subnucleus, causing N(10) to have a much lower pK than N(5) [6a]. Therefore, we can neglect N(10)-inversion and we are left to consider the invertomeric conformations a and c in Fig. 1 as the essential constituents of the equilibrium in solution.

Since N(5) is an active centre of the flavin nucleus [1], and since flavin-dependent biocatalysis might well involve transfer and fixation of large substrate residues at the flavin site, stereochemistry of flavohydroquinone becomes an important problem. This problem has been overlooked to date owing to the fact that, unlike reduced nicotinamide coenzymes, $1,5\text{-Fl}_{\text{red}}R_2$ loses its stereospecificity upon detachment from the apoprotein. Many enzymological data are, however, in favour of stereospecific donation and back-donation of hydrogen or other reducing equivalents (groups) between flavin and substrate [10], and restriction of proton exchange between $\text{Fl}_{\text{red}}H_2$ and environmental water. Obviously, this phenomenon is not due to the apoprotein limiting the accessibility of water to the flavin site, but is inherent in the reduced flavo-coenzyme itself. Consequently, restriction of N-inversion in $1,5\text{-Fl}_{\text{red}}H_2$ needs careful consideration.

2. Methods and Materials. – Based on these facts, we have tried to find conditions which allow one to evaluate the thermodynamic and kinetic parameters of the equilibrium $a \rightleftharpoons d$ (Fig. 1) by means of proton magnetic resonance. We found two methods helpful in slowing the inversion frequency down to the NMR. range:

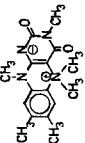
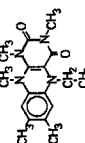
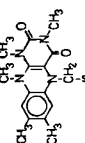
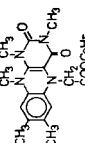
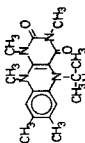
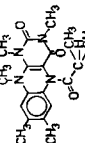
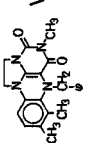
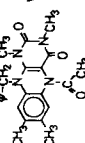
i) The temperature variation method [9]. This method was applied for all compounds of Table 2, dissolved in d_6 -acetone and, in one case, in addition in a 1:1 mixture of d_6 -acetone and CS_2 .

ii) The protonation method [11]. This method was only applicable in the case of the N(5)-benzyl derivative (III) (cf. Table 2), because of its stability against oxidation and its appropriate pK-value. The solvent was a CF_3COOD/D_2O mixture of continuously varied composition. As indicator for a blocked N-inversion we choose the diastereotopism generated by N-chirality at the α -carbon atom of N(10)- and N(5)-alkylated flavohydroquinones. Substituents $-\text{CH}_2-\text{C}_6\text{H}_6$ (III and VIII) and $-\text{CH}_2\text{COOC}_2\text{H}_5$ (IV) could be expected to yield four line AB-systems, whereas for $-\text{CH}_2-\text{CH}_3$ an ABX_3 pattern should be observed. In the case of isopropyl (V) and isobutyryl (VI) residues, the diastereotopism due to arising N-chirality should result in an A_3B_3X -line pattern from the nonequivalent methyl groups.

In earlier work [6a, b], a very strong diastereotopism was observed in the methylene groups of a $(\text{CH}_3\text{CH}_2)_2\text{N}(5)^+$ -centre of an N(5)-quaternized flavohydroquinone ($\delta\omega = 1.85$ ppm) for the non-equivalent methylene hydrogens. Well resolved

Table 2. Thermodynamic parameters for *N*-inversion in different reduced flavins

The table shows the structure formula (col. 1), the solvent (col. 2), the inversion rate *k* (col. 3) at temperature *T* (col. 4), and the corresponding free activation energy $\Delta G^\ddagger$ (col. 5), the enthalpy $\Delta H^\ddagger$ and entropy $\Delta S^\ddagger$ (cols. 6 and 7), the enthalpy $\Delta H^\ddagger$ for $\Delta S^\ddagger = 0$ (col. 8), the chemical shift ω_0 of the analysed signal (col. 9), the difference of the chemical shifts $\delta\omega$ of the proton groups A and B in the NMR. (col. 10), and the coupling constant J_{AB} between them (col. 11). *N*-inversion barriers of 10–15 kcal/mol correspond to inversion frequencies at room temperature between 10^5 – 10^6 Hz.

(1) Compound	(2) Solvent	(3) <i>k</i> [Hz]	(4) <i>T</i> [°C]	(5) $\Delta G^\ddagger$ [kcal/mol]	(6) $\Delta H^\ddagger$ [kcal/mol]	(7) $\Delta S^\ddagger$ [e.u.]	(8) $\Delta H^\ddagger$ ($\Delta S^\ddagger = 0$) [kcal/mol]	(9) ω_0 [ppm]	(10) $\delta\omega$ [Hz]	(11) J_{AB} [Hz]
	I Acetone	170 ± 90	-95	< 8	9	5	< 8	4.33	17 ± 5	-
	II Acetone	167 ± 15	-62	10.0	10.9	3.8	10.1 ± 0.3	3.65	19.5	12.0
	III Acetone	28.0 ± 1.5	-75	10.1	9.6	-2.3	10.1 ± 0.3	4.83	20.0 ± 0.5	12.5 ± 0.5
	Acetone + CS ₂	27.0 ± 1.5	-75	10.1	10.5	-1.8	10.1 ± 0.3	4.75	24.5 ± 0.5	14.5 ± 0.5
	CF ₃ COOD/D ₂ O		28.5	13.0 ± 0.3	-	-	13.0 ± 0.3	4.86	15.0 ± 0.5	12.8 ± 0.2
	IV Acetone	32.3 ± 2.3	-72	10.2	10.2	0.3	10.2 ± 0.3	4.59	19.0 ± 0.5	19.0 ± 1.0
	V Acetone	70.0 ± 11.0	-73	9.9	9.6	-1.6	9.9 ± 0.3	1.17	15 ± 3	7.06 ± 0.10
	VI Acetone	73.0 ± 13.0	-2	13.5	12.5	-4.8	13.5 ± 0.3	1.05	13.0 ± 0.3	6.9 ± 0.1
	VII Acetone	29.0 ± 0.4	+15	14.9	16.1	4.8	14.9 ± 0.3	4.22	22.5 ± 0.5	14.1 ± 0.2
	VIII Acetone	-	-85	< 9	-	-	< 9	4.93 [N(10)-CH ₂] 3.05 [N(5)-CH ₃]	17 ± 3	16 ± 3

multiplets could, therefore, be predicted for the N-monosubstituted analogues and even for the β -centre in the N(5)-CO-CH(CH₃)₂ residue in the case of inversion being blocked by protonation or freezing.

Indicative for a blocked ring inversion, on the other hand, would be a splitting of the N(5)-(CH₃)₂ groups in I, yielding one line for the axial and another for the equatorial -CH₃ group. In the case of asymmetric substitution at N(5) (R + H, or R + non-bonding pair), freezing of N-inversion and, in addition, ring inversion would stabilize the less hindered conformer preferably, and would thus split the above-mentioned multiplets originating from the diastereotopism in two line sets of non-stoichiometrically different intensity. Freezing of the rotation around the N(5)-C(4 α) axis in the N(5)-acetyl (VIII) or the N(5)-isobutyryl (VI) derivatives should also result in a splitting of the acetyl and isobutyryl NMR. signals corresponding to the different rotamers.

The measurements were done with a Varian A-60A spectrometer and a Varian V6040 temperature control unit. The calibration of the temperature scale was done with CH₃OH and (CH₃OH)₂. At each temperature setting the field homogeneity of the apparatus was readjusted, thus ensuring a resolution of better than 1 Hz. Special care was taken to keep the cooling nitrogen flow constant during a particular temperature setting. A temperature constancy of the order of 1° could be obtained for short times (~1 h).

Since even a small contribution of paramagnetic flavin radical produces a broadening of the NMR. lines, a small amount of Zn powder was added of the CF₃COOD/D₂O solution whenever necessary to avoid partial oxidation of the reduced flavin species during the measurements.

The desired model flavohydroquinones have been synthesized as follows:

3,5,5-Trimethyl-1,5-dihydrolumiflavin (I) was obtained by reductive methylation of 3-methyl-lumiflavin as described elsewhere [6a].

1,3-Dimethyl-5-alkyl-1,5-dihydrolumiflavins (II-V): A solution of 1,3-dimethyl-1,5-dihydrolumiflavin was prepared by suspending 0.4 g (1 mmol) of 1,3-dimethylalumiflavinium perchlorate [12] in 20 ml tetrachloroethane, and shaking this in a dropping funnel with 20 ml of a saturated sodium chloride solution containing 0.4 g sodium dithionite and 0.5 g hydrogen carbonate until reduction was complete. This solution (organic phase) was transferred anaerobically to an argon-flushed vessel containing 2.0 g of dry potassium carbonate, 0.2 g of sodium dithionite and 0.1 ml ethyl-diisopropyl amine. The mixture was brought under stirring to the temperature given below, and a 20fold excess of the alkylating agent in 2 ml tetrachloroethane was added over 30 min. The reaction course was followed by thin layer chromatography in benzene/diisopropyl ether/ethanol 7:2:1 on silica plates until no more starting material was present. The reaction mixture was then washed with water, 2N acetic acid, hydrogen carbonate and water, the organic phase was dried over magnesium sulphate and the solvent distilled off under reduced pressure. The oily residue was dissolved in benzene, then treated with charcoal and the product separated by fractional crystallization from benzene/hexane at 5°. For analytical purposes the crude product was recrystallized as mentioned below. The yields were 40 to 70%. UV. and IR. spectra of II-V were in agreement with the data reported by Dudley *et al.* [12] for this class of dihydroflavins.

1,3-Dimethyl-5-ethyl-1,5-dihydrolumiflavin (II) was obtained with diethylsulphate at 70°, and recrystallization from benzene/pentane. M.p. 199–201°. NMR. (acetone-d₆): δ = 6.72 (1H, s, C(6)-H), 6.69 (1H, s, C(9)-H), 3.65 (2H, q, J = 7 Hz, N(5)-CH₂-), 3.37 (3H, s, N(3)-CH₃), 3.20 (6H, N(1)-CH₃ + N(10)-CH₃), 2.15 ppm (6H, C(7)-CH₃ + C(8)-CH₃).

1,3-Dimethyl-5-benzyl-1,5-dihydro-lumiflavin (III) was obtained with benzylbromide at 40° and recrystallized from benzene/pentane. M.p. 195–197°. NMR. (CS₂): δ = 7.25 to 6.78 (5H, m, N(5)-C-C₆H₅), 6.66 (1H, s, C(6)-H), 6.38 (1H, s, C(9)-H), 4.65 (2H, s, N(5)-CH₂-), 3.19 (6H, N(3)-CH₃ + N(1)-CH₃), 3.16 (3H, s, N(10)-CH₃), 2.17 + 2.12 ppm (6H, C(7)-CH₃ + C(8)-CH₃).

1,3-Dimethyl-5-carbethoxymethyl-1,5-dihydrolumiflavin (IV) was synthesized with ethyl-bromoacetate at 50° and recrystallized from chloroform/pentane. M.p. 177–180°. NMR. (acetone-

d_0): $\delta = 6.75$ (1H, s, C(6)—H), 6.70 (1H, s, C(9)—H), 4.58 (2H, s, N(5)—CH₂), 3.42 (3H, s, N(3)—CH₃), 3.30 (3H, s, N(1)—CH₃), 3.21 (3H, s, N(10)—CH₃), 2.15 + 2.10 ppm (6H, C(7)—CH₃ + C(8)—CH₃).

1,3-Dimethyl-5-isopropyl-1,5-dihydroalumiflavin (V) was obtained with isopropyl iodide at 70° and recrystallized from benzene/pentane. M.p. 190–195°. NMR. (CDCl₃): $\delta = 6.82$ (1H, s, C(6)—H), 6.68 (1H, s, C(9)—H), 3.64 (1H, q, $J = 6.5$ Hz, N(5), N(5)—CH—H), 3.41 (3H, s, N(3)—CH₃), 3.38 (3H, s, N(1)—CH₃), 3.25 (3H, s, N(10)—CH₃), 2.20 (6H, C(7)—CH₃ + C(8)—CH₃), 2.22 ppm (6H, d, $J = 6.5$ Hz, N(5)—CH—(CH₃)₂).

1,3-Dimethyl-5-isobutyryl-1,5-dihydroalumiflavin (VI). To a solution of 0.4 g (1 mmol) 1,3-dimethyl-lumiflavinium perchlorate [12] in 10 ml of a 1:1 mixture of isobutyryl anhydride and isobutyric acid were added small portions of zinc dust at 90° until the red colour due to intermediate formation of radical cation disappeared. The course of the reaction was followed by thin layer chromatography (cf. above). After completion of the reaction the zinc salts were filtered off, and water was added to the filtrate in order to hydrolyze excess anhydride. This solution was then extracted with chloroform, the organic phase was extensively washed with buffer pH 7, dried over magnesium sulphate, and the solvent distilled off under reduced pressure. The oily residue was crystallized from chloroform/diisopropylether to yield 0.13 g (85%) of VI. Recrystallization from tetrahydrofuran/diisopropylether yields the pure VI, M.p. 202–204°. NMR. (acetone- d_6): $\delta = 7.44$ (1H, s, C(6)—H), 7.13 (1H, s, C(9)—H), 3.54 (6H, N(1)—CH₃ + N(3)—CH₃), 3.29 (3H, s, N(10)—CH₃), 2.95 (1H, q, $J = 6.5$ Hz, N(5)—CO—CR₂H), 2.28 (6H, C(7)—CH₃ + C(8)—CH₃), 1.07 ppm (6H, d, $J = 6.5$ Hz, N(5)—CO—CH—(CH₃)₂).

1,10-Ethano-3,6,7-trimethyl-5-benzyl-1,5-dihydroalloxazine (VII) was prepared from 1,10-ethano-3,6,7-trimethyl-alloxazinium perchlorate by photochemical benzylation with phenylacetate as will be described elsewhere [13].

1,3-Dimethyl-5-acetyl-10-benzyl-1,5-dihydroalloxazine (VIII) was synthesized as described elsewhere [14].

The commonly used method of evaluating coalescence spectra is the coalescence point approach [15], where the inversion rate is determined at only one temperature, the coalescence temperature. In this work the line shapes were analysed in order to obtain inversion rates at different temperatures.

The theoretical line shapes, derived according to *Bloch-McConnell's* theory, are known for a system of two uncoupled proton groups and for an AB-system [15]. The line shapes of the other occurring proton systems (ABX₃ and A₃B₃X) were derived analogously by one of us (L. T.). The free parameters of the theoretical line shapes were determined in such a way as to approximate best the measured spectra. This optimization was done by a computer program: the sum of the squared deviations of the theoretical line shape from the measured one was minimized, using three different minimization algorithms [16]. The free parameters are:

a) a linear background, b) the centre of gravity of the line ω_0 , c) the intensity of the line h_0 , d) the transversal relaxation time T_2 , e) the inversion frequency k , f) the coupling constants J_{AB} , J_{AX} , g) the difference of the chemical shifts of the two coalescing proton groups $\delta\omega$.

Since the instrumental resolution was better than 1 Hz during the measurements, it was not necessary to convolute the theoretical line shape with the resolution curve.

The evaluation showed that independently of the actual temperature, the minimization converged rapidly for the background, ω_0 and h_0 . For the remaining parameters there were essentially three temperature regions:

i) at temperatures higher than the coalescence temperature T_C , i.e. at high inversion frequencies, rapid convergence is only obtained for T_2 , whereas $\delta\omega$, J_{AB} , and k have large errors;

- ii) at temperatures below T_C , *i.e.* at blocked inversion, the hyper area of the parameters T_2 , J_{AB} , and $\delta\omega$ has a sharp minimum, whereas k has again a large error;
- iii) at temperatures around T_C , a very sharp minimum exists for k , which is nearly independent of the other parameters.

Since it turned out from the measurements below T_C , that J_{AB} and $\delta\omega$ are almost constant within a temperature range of about 30° below T_C , these parameters were, whenever possible, determined at completely blocked inversion ($T \approx T_C - 30^\circ$) and then kept fixed in the fits of the higher temperature measurements. The initial value of T_2 was normally taken from the converted line width of the C(6) proton, since it turned out during our measurements that the relaxations of this proton and the protons of the N(5) substituents behave similarly at room temperature.

Table 2 shows the inversion frequency k (column 3) at the temperature T (column 4) near the coalescence point. The errors are due to statistical and minimization errors and to the uncertainty of the temperature of $\pm 5^\circ$. Additional uncertainties are introduced in the case of protonation of the N(5)-benzyl derivative (III) by errors in the determination of the CF_3COOD/D_2O mixture, the error of the pK value, and the error of the acidity function.

From the inversion frequencies k , the thermodynamical terms, which govern the inversion process, can be obtained. The *Eyring* equation yields the free energy of activation $\Delta G^\ddagger$:

$$k = \frac{kT}{h} e^{-\Delta G^\ddagger/RT}. \quad (1)$$

The free energy of activation is temperature-dependent:

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger. \quad (2)$$

The enthalpy of activation $\Delta H^\ddagger$ is temperature-independent and therefore a direct measure of the potential barrier of the process under consideration [9]. Table 2 shows the free energy of activation $\Delta G^\ddagger$ (column 5), resulting from the inversion frequency k (column 3) at the temperature T (column 4), for the one particular measurement near the coalescent point.

In order to obtain the enthalpy of activation and entropy, the $\Delta G^\ddagger$ in Eq. (1) was replaced by Eq. (2). Each compound was measured at five or more different temperatures in a temperature range of about $\pm 30^\circ$ around the coalescence temperature. We could therefore determine $\Delta H^\ddagger$ and $\Delta S^\ddagger$ by a numeric minimization [16b] of the squared deviations of the measured k values from the calculated ones, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ being the free parameters. The final $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values are listed in Table 2, columns 6 and 7.

The $\Delta S^\ddagger$ values are not very well determined, because only a relatively small temperature range of about 60° is covered by the method used. Therefore a further minimization was done keeping $\Delta S^\ddagger$ equal to zero. The $\Delta H^\ddagger$ values obtained from this calculation are listed in Table 2, column 8. The errors quoted result from the uncertainties of the k values and the temperature. The $\Delta H^\ddagger$ values are almost identical with those of column 5, which again shows that the method is most sensitive to the inversion frequencies near the coalescence point.

. Results and Discussion. – Since the aim of this work was the investigation of the N-inversion, it was necessary to make sure that the observed rates were really due to N-inversion and not to ring inversion or rotation.

The *ring inversion process* was investigated by the N(5)-dimethyl cation derivative (I) dissolved in acetone. Even at -107° , the coalescence point was not reached. However, a strong selective broadening of the N(5)- $(\text{CH}_3)_2$ signal was observed. In piperidine derivatives the ring inversion barrier is reduced from 10–12 kcal/mol to less than 8 kcal/mol by introducing an sp^2 -centre ($\text{C}=\text{O}$ or $\text{C}=\text{CH}_2$) in para position [9] [17] [18] [19].

In cases of our compounds II–VII, where the diastereotopism of the 5α -centre yields an AB- or ABX₃-line pattern upon freezing, contributions of ring inversion are eliminated by the evaluation procedure, since freezing of ring inversion would result in an additional splitting (or at least broadening) of axial and equatorial proton groups.

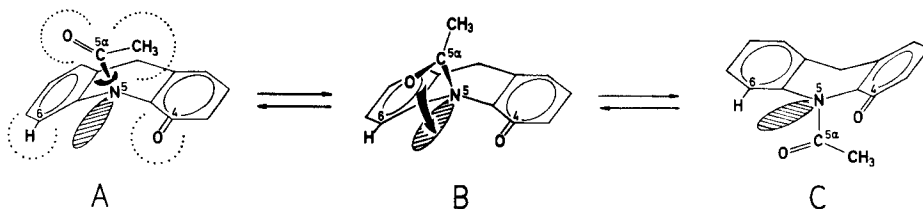


Fig. 4. Schematic representation of the combined inversion processes of VI involving rotation around the N(5)–C(5 α) bond (A)

followed by pyramidal inversion on the N(5)-centre (B) and subsequent fast ring inversion (C). Note that process (A) must precede (B)

The *rotation* of the N(5) substituent around the N(5)–C(5 α) axis was investigated in the N(5)-acetyl derivative (VIII). Freezing the rotation in a way indicated in Figs. 4A and C, *i.e.* the methyl group on the side of the aromatic ring A and the oxygen of the pyrimidine side B of the flavin nucleus and *vice versa*, would cause a selective broadening or even a splitting of the N(5)-acetyl proton signal. These two positions are, at least in the crystal, the most stable positions [5c, d]. Neither ring inversion nor N-inversion would eliminate such a chemical inequivalence. The population of both rotamers is expected to be roughly equal since the steric hindrance is about the same for both.

Compound VIII was measured in acetone at temperatures down to -107° . Neither selective broadening nor splitting of the N(5)-acetyl proton signal could be observed. This indicates that the rotation barrier is considerably below 9 kcal/mol. A normal amide resonance would cause rotation barriers of 14–18 kcal/mol [20]. So we conclude that an amide resonance in the N(5)-acetyl derivative (VIII) is negligible. This was already expected from the crystallographic data [5c, d], where it was found that the N(5)-centre was highly pyramidal, the out-of-plane angle [*i.e.* the angle between the N(5)–C(5 α) bond and the plane defined by the N(5)–C(4a) and N(5)–C(5a) bonds] being $\sim 20^\circ$ (Table 1).

All other 5-substituents in II–VII can be expected to have rotational barriers not greater than that of the N(5)-acetyl compound in VIII, except for VI (see below).

Hence, freezing of rotation can be excluded, as well as freezing of ring inversion, as the reason for spectral changes in the temperature range down to -110° for all compounds measured except VI and VII (see below).

The *N*-inversion was investigated with the N(5)-ethyl-(II), N(5)-benzyl-(III), N(5)-carboxymethyl-(IV), N(5)-isopropyl-(V), N(5)-isobutyryl-(VI), N(5)-benzyl-N(1),N(10)-ethanoisoflavin (VII), and the N(5)-acetyl-N(10)-benzyl (VIII) derivatives. The only NMR. signals which changed with temperature were those of the N-methylene protons (compounds II–VI, VII, and VIII) or those of the isopropyl protons (compounds V and VI). Fig. 5 shows the typical temperature dependence of the line shape of the N(5)-methylene protons in the N(5)-benzyl derivative (III) in acetone solution.

The results are shown in Table 2, lines 2–10. In the case of the N(5)-ethyl derivative (II), the resolution at low temperatures was not good enough to determine J_{AB} and $\delta\omega$ from the spectra. Therefore the values obtained from the N(5)-benzyl derivative (III) in acetone solution (Table 2, line 3) were used for the calculations. The coalescence point could not be reached for the N(10)-methylene signal in the N(5)-acetyl-N(10)-benzyl derivative (VIII). The uncertainty of the resulting $\delta\omega$ and J_{AB} was therefore extended to cover the corresponding values of the N(5)-benzyl derivative (III) in acetone solution.

The enthalpy of activation $\Delta H^\ddagger$ (Table 2, column 8) is equal, within the error units, for the compounds II, III, IV, and V, and has a value of $\Delta H^\ddagger = 10.1$ kcal/mol in non-polar solvents (acetone and acetone- CS_2 mixture). The influence of the mass of the N(5) substituent on N(5)-inversion seems, therefore, to be negligible. Furthermore, the steric effects exerted by the C(4)-oxygen and the C(6)-hydrogen on the various N(5)-substituents of different size appear to be negligible for the different substituents. This may also be interpreted in such a way that the rotational mobility around the N(5)–C(5 α) axis is always high enough to allow for an optimal transition position of the N(5)-ligand for N-inversion. The free activation entropy $\Delta S^\ddagger$ for these four compounds (Table 2, column 7, lines 2–7) has values between -2.3 e.u. and 3.8 e.u. Since for all four compounds the same $\Delta S^\ddagger$ is expected, this range indicates the uncertainty of the $\Delta S^\ddagger$ determination.

The N-inversion barriers of nitrogen bound in two different sixmembered rings are given for comparison. The N-methyl-piperidone-4 in CH_2Cl_2 has an enthalpy of activation $\Delta H^\ddagger = 8.6$ kcal/mol [21], whereas for the 2-alkyl-2,3-dihydro-1H-benz[de]isoquinoline $\Delta H^\ddagger = 9.7$ kcal/mol has been measured [22].

The free activation enthalpies for VI and VII (Table 2) are about 4 kcal/mol higher than those of the preceding compounds (II–V).

This difference appears to be significant, since it concerns just those two model compounds where substituent overcrowding must be expected to become relevant for conformational stability. In compound VI the bulkiness of the 5 α -isopropyl residue can be expected to hinder rotation around the N(5)–CO bond, while introduction of a peri- CH_3 in VII should affect ring inversion. This is in agreement with the behaviour of *Stuart-Briegleb* molecular models.

As shown in Fig. 4B, rotation and N-inversion in VI might be strictly coupled, as well as N-inversion and ring inversion in VII, thus explaining the higher enthalpy level. This process corresponds to a direct transition a–c in Fig. 1.

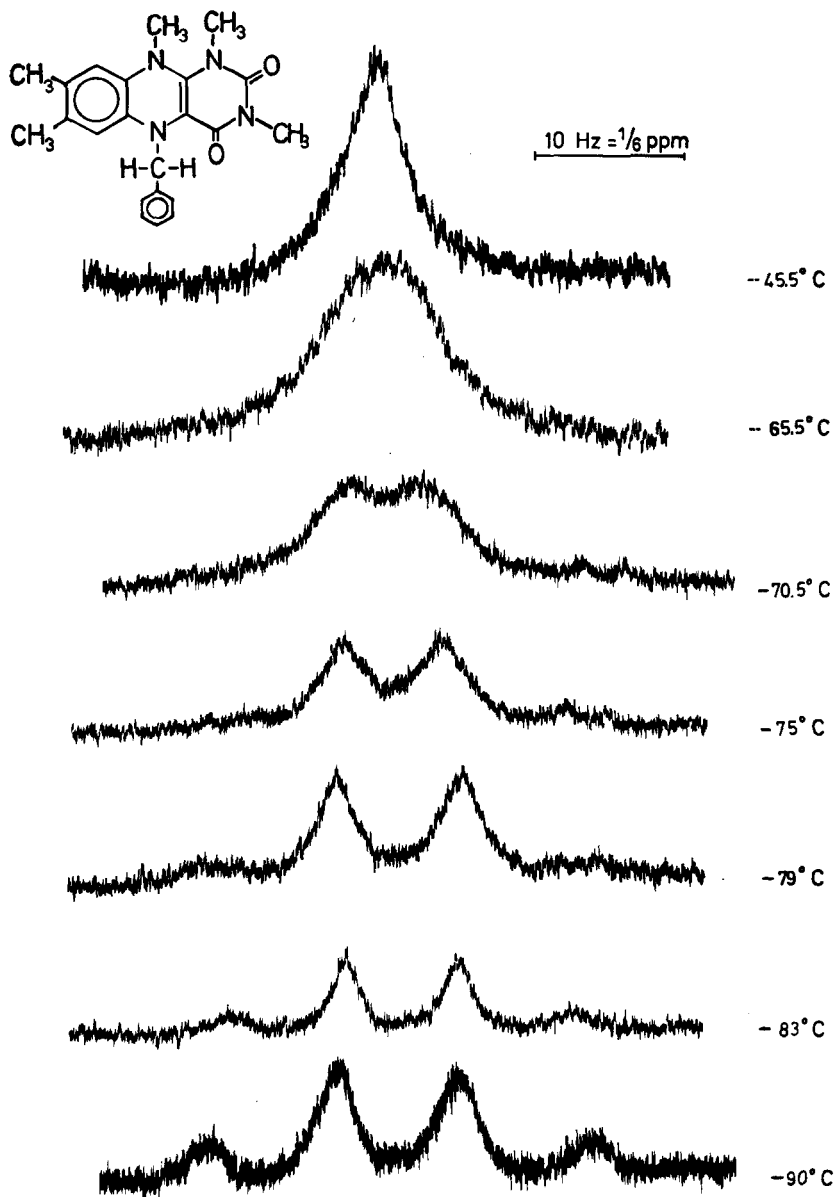


Fig. 5. Line shape of the N(5)-methylene NMR. signal of the N(5)-benzyl derivative III in d_6 -acetone at different temperatures

The influence of the solvent on the N-inversion was investigated with the N(5)-benzyl derivative III. As seen in Table 2, lines 3 and 4, no influence is observed as long as the solvent is non-polar. Only $\delta\omega$ and J_{AB} are affected. In aqueous solution, however, an important change is observed, as seen in Table 2, line 5. Here the N-inver-

sion was measured by the protonation method, dissolving the compound III in absolute CF_3COOD and diluting successively with D_2O . The pK of III was determined spectrophotometrically as 0.85 ± 0.05 , using a calibrated *Hammett* set. The acidity function for solutions of CF_3COOH in water was taken from *Randles & Tedder* [23]. The measurement at room temperature yields $\Delta H^\ddagger = 13.0$ kcal/mol, a value which is higher by 2.9 kcal/mol than that for the same compound in acetone solution. This increase may be due to the hydration of the N(5) electron lone pair, as was also observed in dibenzylmethylaniline [24] [11a].

Finally the N(5)-acetyl-N(10)-benzyl compound VIII also yielded information about the N(10)-centre. Only an upper limit of the N(10)-inversion barrier by the value of 9 kcal/mol can be given from the selective broadening of the $10\alpha\text{-CH}_2$ peak at $\sim -100^\circ$. This confirms the above-mentioned fact that the basicity of the N(10)-centre is less than that of the N(5)-centre. Furthermore, it indicates that the N(10)-centre is less pyramidal than the N(5)-centre.

4. Conclusions. – The conformational analysis of flavohydroquinone was hitherto confined to the crystalline state [5]. From the X-ray data it cannot be decided whether or not the bending of this molecule depends mostly on environmental (lattice) forces, steric intramolecular overcrowding, or electronic properties of the heteroaromatic system involved. The present study on liquid solutions allows one to determine electronic properties, *i.e.* 'antiaromaticity', as the main obstacle for coplanarity. Steric overcrowding proves to be of minor importance, whereas environmental forces can be largely neglected.

In agreement with the crystal data, N(5)-acyl substituents do not show an essential amide resonance. The question seems of potential relevance in lipoamide-free pyruvate oxidase systems, where the possibility of an oxidative transfer of 'active acetyl' from thiamine to flavin should at least be discussed [25].

Moreover, it can be seen from the present data that the conformational energy provided by an apoprotein might well be sufficient to 'freeze' a specific flavohydroquinone configuration, inducing N-chirality at the active site and, in consequence, a stereospecificity of 'hydrogen transfer' (or group transfer) from substrate to flavin. Several indications for such a stereospecificity are found in the literature [10] [26]. Such effects have been hitherto interpreted as characteristics of a given apoprotein, which would limit water accessibility and, by consequence, proton exchange. Instead, we might have to consider an N(5)R-group of a protein-bound flavin as a centre of preserved chirality, and, if $\text{R} = \text{H}$, slow proton exchange with the environment, by virtue of the coenzyme itself. Clearly, the chirality is lost not only upon oxidation, but also upon detachment of the reduced coenzyme from the protein, and this is the very difference between flavin and nicotinamide in the present stereochemical context.

Since we assume that, quite generally, flavin dependent dehydrogenations involve formation and decay of covalent flavin-substrate complexes, we want to emphasize the possibility of stable N(5)-centres of flavin chirality.

Finally, it has been pointed out that all bent conformations of flavohydroquinone (Fig. 1) can interconvert without ever swinging through the 'all-coplanar' state. This state is, owing to its 'antiaromatic character', at an energy maximum, which can,

presumably, only be lowered by a change of spin state from singlet to triplet. In contact with paramagnetic species, *i.e.* heavy metal ions or molecular oxygen, or with aromatic acceptor molecules, *e.g.* flavoquinone, this transition may become allowed. Here is an obvious possibility for magnetic steering of biological oxidation and a reasonable explanation for the 'O₂-activating' properties of flavohydroquinone. Such enforced flattening of reduced flavin will enhance its reducing power, for 1e⁻- as well as 2e⁻-transfers, while enforced bending of flavoquinone will enhance its oxidizing power (and 2e⁻-transfer selectively). Hence, conformational strains induced by the protein will govern the actual redox potential of flavin in a kind of amplifier effect, which might explain the wide range of flavin 2e⁻-potentials found in flavo-proteins. It could, at the same time, decide over the 2e⁻-potential being split into upper and lower 1e⁻-potentials [1], which implies the 'switch' from dehydrogenation to electron transfer, the most important aim of flavins in biology.

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64. Metastabile, basische Bleisalze des 2,4,6-Trinitroresorcins

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Zusammenfassung. Beim Zusammengiessen einer alkalischen Lösung von 2,4,6-Trinitroresorcin mit einer Bleilösung entstehen zwei metastabile Bodenkörper, und zwar bei pH 5–9 eine neue Modifikation (I) des bereits bekannten $\text{Pb}_2(\text{OH})_2\text{Tric}$ (Tric = Anion des 2,4,6-Trinitroresorcins), und bei pH 10–12,5 $\text{Pb}_7(\text{OH})_{10}\text{Tric}_2$ (II). I wandelt sich in die bekannte stabile Form um, II reagiert bis pH 11 zu $\text{Pb}_5(\text{OH})_6\text{Tric}_2$, darüber zu $\text{Pb}_{13}\text{O}_7(\text{OH})_{10}\text{Tric}_2$.

1. Einleitung. – Bei der Untersuchung der Bleisalze des 2,4,6-Trinitroresorcins [1] beobachteten wir, dass beim Zusammengiessen einer Lösung von 2,4,6-Trinitroresorcin in Natronlauge und einer Bleilösung ein gelber bis rotoranger, flockiger Niederschlag entsteht, der innerhalb einiger Tage bis Wochen sich je nach dem pH der Lösung in eines der bereits beschriebenen Salze des 2,4,6-Trinitroresorcins umwandelt.

Nach unseren Erfahrungen handelt es sich bei diesem metastabilen Niederschlag je nach den Fällungsbedingungen entweder um eine zweite Modifikation von $\text{Pb}_2(\text{OH})_2\text{Tric}$ (Tric = Anion des 2,4,6-Trinitroresorcins) oder um eine Verbindung von der Formel $\text{Pb}_7(\text{OH})_{10}\text{Tric}_2$. Die schon bekannte stabile Form von $\text{Pb}_2(\text{OH})_2\text{Tric}$ bezeichnen wir als α -, die metastabile Form als β -Modifikation.

2. Herstellungsvorschriften. – Beide Verbindungen kann man aus Bleinitrat- oder Bleiperchloratlösungen erhalten, wobei man mit Vorteil bei konstantem pH arbeitet und das Reaktionsgemisch zur Beschleunigung der Umsetzung rührt. Wegen der sonst eintretenden Bildung von basischem Bleicarbonat ist nur CO_2 -freies Wasser zu verwenden und das Luft- CO_2 strikte auszuschliessen.

Wir verwendeten: Als *Reaktionsgefäss* ein dickwandiges 300-ml-Becherglas mit plangeschliffenem Rand und einem Deckel mit Durchführungen für den Propellerrührer und die Glaselektrode, einer Zuführung für Salpetersäure von der Motorburette her sowie zu- und Ableitung für Stickstoff.

Als *pH-Stat* ein Titrator Typ TTT 1c der Firma Radiometer mit angekoppelter Motorburette Typ SBU 1a und einer Glaselektrode Metrohm Typ UX.

0,2M $\text{Pb}(\text{NO}_3)_2$ -Lösung: 66,2 g $\text{Pb}(\text{NO}_3)_2$ p.a. werden zu 1000 ml gelöst.

0,2M $\text{Pb}(\text{ClO}_4)_2$ -Lösung: 66,2 g $\text{Pb}(\text{NO}_3)_2$ p.a. werden in einer Platinschale vorsichtig mit 60 g 70proz. HClO_4 abgeraucht. Den Rückstand löst man zu 1000 ml.