The Crystal Structure of 10-Methylisoalloxazine Hydrobromide Dihydrate*

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The crystal structure of 10-methylisoalloxazine hydrobromide dihydrate, $C_{10}H_9N_4O_2.HBr.2H_2O$, has monoclinic symmetry $P2_1/c$. Unit-cell constants are $a = 9.42 \pm 0.01$, $b = 11.81 \pm 0.01$, $c = 13.64 \pm 0.02$ Å, $\beta = 118.27 \pm 0.06°$. There are four molecules in the unit cell. All components of the structure lie in layers, with the layers grouped into pairs by hydrogen bonding. The nearly planar 10-methylisoalloxazine molecule is protonated at N(1) and bowed slightly on its long axis. N(1) and N(3) are hydrogen bond donors to water and bromide respectively; CO(4) is a hydrogen bond receptor.

Introduction

Riboflavin (I, with R = D-ribityl and R' = methyl)

(I)
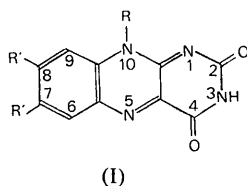
is coenzyme for a large family of flavoproteins, catalysts in biological redox reactions. The coenzyme, usually along with a metal atom, is present at the enzymic active site (Hemmerich, Müller & Ehrenberg, 1965), and binding to the metal through N[5] and CO[4]† is proposed (Hemmerich & Fallab, 1958; Hemmerich, 1964). It has been suggested that charge-transfer bonding between flavin and the substrate may be catalytically important (Szent-Gyorgyi, 1960). To clarify the mode of bonding of riboflavin to metal and to substrate, we have begun studying simple flavins in various molecular complexes. This paper, reporting the structure of 10-methylisoalloxazine hydrobromide dihydrate (I, R = methyl, R' = hydrogen), is the first of the series.

Experimental data

Crystals were grown from hot concentrated HBr saturated with 10-methylisoalloxazine. Cooling produced transparent yellow needle-like gems with [010] as the needle axis.

The reciprocal-lattice symmetry and systematic absences, determined from the zero and first layer Weis-

senberg photographs of a crystal rotating about **b** and an 0*kl* precession picture, indicated that the space group is the monoclinic group $P2_1/c$. Preliminary lattice constants determined from the *b* axis rotation photograph and the *h*0*l* Weissenberg photograph were refined by the use of 2θ measurements made with a Picker diffractometer. The refined values are $a = 9.42 \pm 0.01$, $b = 11.81 \pm 0.01$, $c = 13.64 \pm 0.02$ Å, and $\beta = 118.27 \pm 0.06°$. The density was determined to be 1.74 g.cm^{-3} by flotation techniques, whereas it is calculated to be 1.72 g.cm^{-3} with four formula units per cell.

Intensity data were measured on a Picker card-controlled four circle diffractometer with Zr-filtered Mo $K\alpha$ radiation. Reflections with $3° \leq 2\theta \leq 40°$ were scanned in the 2θ mode at a rate of 1° per minute, with a scan range of 2°. A twenty second background count was recorded on either side of the scan range. A pulse height analyzer adjusted to record about 90% of the Mo $K\alpha$ pulse distribution was used in conjunction with a scintillation counter as a radiation detector. Two standards were determined approximately every four hours and served to measure any variation in the crystal or instrument. The standards decreased by an average of about 10% with time. There was no significant change in the appearance of reflections, although the crystal became slightly clouded. The crystal used had approximate dimensions $0.54 \times 0.28 \times 0.42$ mm. Absorption and extinction corrections were not applied.

Altogether, 2354 reflections were scanned. Each reflection was assigned a standard deviation based on a combination of standard counting statistics in the integrated scan and in the background plus an uncertainty of 3% in the time-dependent scale factor. Squares of structure factor amplitudes, $|F_o|^2$, and their standard deviations were derived from the net intensities by application of the Lorentz and polarization (Lp) factors.

Any reflection for which the net value of $|F_o|^2$ was less than twice the corresponding standard deviation was considered to be below an observational threshold.

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* Previously reported in part: Trus & Fritchie, *Chem. Comm.* (1968), p. 1486.

† Brackets are used to refer to the chemical numbering system, (I). Parentheses refer to the crystallographic system, Fig. 1.

The final data set contained 668 of these 'unobserved' reflections and 1686 observed reflections. For each of the observed reflections a standard deviation in $|F_o|$ was derived by the standard propagation-of-error equation. Similar standard deviations were derived for the other reflections by use of the threshold value of $|F_o|, 2\sigma_F$. The unobserved reflections were omitted from all calculations, except for a comparison with their final calculated values.

Structure solution and refinement

The structure was solved by interpretation of the three-dimensional Patterson function by the heavy atom method. A first Fourier synthesis, with $R(=\Sigma |F_o - F_c|/\Sigma |F_o|) = 40\%$ based on signs determined from the location of the bromine atom alone and by use of all of the observed data, permitted location of a clear napthalene-like structure, addition of which lowered R to 36%. The two oxygen atoms and the methyl carbon atom were found in a second Fourier synthesis and lowered R to 31% after isotropic least-squares refinement (Hughes, 1941) of all the atoms. Addition of the four remaining ring carbon atoms produced an R of 25% after refinement. Location of the two oxygen atoms of the water molecules lowered R to 17%. After anisotropic refinement of bromine alone for three cycles, all atoms were refined anisotropically, lowering R to 7·3%. The aromatic hydrogen atoms and two water hydrogen atoms were found on a difference map calculated at this stage. With these added, the heavy atoms refined further to $R = 6·2\%$. The methyl hydrogen atoms were then found on a difference map. Refinement of all parameters except the hydrogen temperature

Table 1. *Positional and isotropic thermal parameters*

Standard deviations are given in parentheses.

	x^*	y^*	z^*	B
Br(1)	29169 (6)	12810 (4)	3061 (4)	—
N(2)	1910 (4)	3261 (4)	2263 (3)	—
C(3)	2863 (6)	4239 (4)	2686 (4)	—
N(4)	4459 (5)	4066 (3)	3449 (3)	—
C(5)	5179 (5)	3029 (4)	3882 (4)	—
C(6)	4115 (5)	2026 (4)	3398 (3)	—
N(7)	4766 (4)	1036 (3)	3736 (3)	—
C(8)	3810 (6)	100 (4)	3276 (4)	—
C(9)	4494 (6)	−987 (4)	3609 (4)	—
C(10)	3592 (6)	−1915 (4)	3139 (4)	—
C(11)	1966 (5)	−1806 (3)	2306 (3)	—
C(12)	1248 (6)	−773 (4)	1994 (4)	—
C(13)	2178 (5)	214 (4)	2478 (3)	—
N(14)	1516 (4)	1286 (3)	2177 (3)	—
C(15)	2473 (5)	2189 (4)	2597 (4)	—
C(16)	−213 (6)	1420 (4)	1335 (4)	—
O(17)	2282 (5)	5168 (3)	2377 (3)	—
O(18)	6580 (4)	2953 (3)	4578 (3)	—
O(19)	−1136 (4)	3899 (3)	385 (3)	—
O(20)	1106 (5)	3758 (3)	−586 (3)	—
H(21)	98 (6)	331 (4)	182 (4)	5·3 (1·4) Å²
H(22)	530 (6)	467 (4)	393 (4)	4·6 (1·3)
H(23)	559 (5)	−103 (4)	420 (3)	3·4 (1·1)
H(24)	394 (5)	−258 (3)	334 (3)	3·4 (1·1)
H(25)	104 (6)	−259 (4)	181 (4)	5·4 (1·4)
H(26)	15 (5)	−65 (3)	143 (3)	3·0 (1·1)
H(27)	−83 (6)	95 (4)	152 (4)	5·6 (1·4)
H(28)	−35 (8)	134 (5)	72 (5)	8·0 (1·6)
H(29)	−60 (6)	215 (4)	144 (4)	4·9 (1·3)
H(30)	−128 (6)	456 (4)	45 (4)	4·1 (1·3)
H(31)	−222 (7)	367 (5)	0 (5)	8·9 (1·6)
H(32)	34 (5)	377 (4)	−39 (4)	3·8 (1·2)
H(33)	159 (7)	292 (6)	−30 (5)	8·5 (1·6)

* Positional parameters and standard deviations multiplied by 10^5 for bromine, 10^3 for hydrogen and 10^4 for remaining atoms.

Table 2. *Anisotropic thermal parameters*

Standard deviations are given in parentheses. Because no absorption correction was made, systematic errors may be present which exceed the usual statistical errors. The thermal expression has the form

$$-[B_{11}h^2 + B_{22}k^2 + B_{33}l^2 + B_{12}hk + B_{13}hl + B_{23}kl].$$

Parameters are multiplied by 10^5 for bromine and 10^4 for other atoms.

	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
Br(1)	1193 (6)	616 (4)	561 (3)	103 (11)	757 (6)	2 (8)
N(2)	95 (6)	51 (3)	48 (3)	21 (7)	36 (6)	7 (5)
C(3)	151 (7)	40 (3)	54 (3)	−2 (9)	99 (7)	−6 (6)
N(4)	103 (6)	50 (3)	51 (3)	−24 (7)	52 (6)	−15 (5)
C(5)	84 (6)	59 (4)	41 (3)	−18 (9)	38 (6)	−3 (6)
C(6)	114 (6)	43 (4)	35 (3)	2 (8)	71 (6)	−4 (5)
N(7)	96 (5)	59 (3)	37 (2)	8 (7)	50 (5)	4 (5)
C(8)	120 (7)	46 (4)	43 (3)	6 (9)	75 (6)	4 (6)
C(9)	125 (7)	59 (4)	45 (3)	20 (9)	58 (7)	11 (6)
C(10)	164 (8)	50 (4)	62 (3)	30 (10)	104 (8)	20 (6)
C(11)	179 (8)	52 (4)	59 (3)	−9 (10)	106 (8)	−7 (7)
C(12)	108 (7)	63 (4)	52 (3)	−12 (10)	64 (7)	7 (6)
C(13)	114 (6)	36 (3)	36 (3)	−3 (8)	67 (6)	2 (5)
N(14)	89 (5)	51 (3)	39 (2)	−10 (7)	51 (5)	2 (5)
C(15)	108 (6)	57 (4)	37 (3)	−5 (9)	60 (6)	3 (6)
C(16)	87 (6)	64 (4)	71 (4)	6 (10)	40 (8)	9 (7)
O(17)	171 (6)	49 (3)	84 (3)	8 (7)	68 (7)	20 (5)
O(18)	109 (5)	61 (3)	71 (3)	−19 (7)	23 (6)	−1 (5)
O(19)	136 (6)	56 (3)	84 (3)	4 (7)	52 (6)	21 (5)
O(20)	185 (6)	79 (3)	107 (3)	24 (9)	139 (6)	9 (6)

Table 3. *Structure factors*

Each group of three columns contains *l*, $10|F_o|$ and $10F_c$, and is headed by the values of *h* and *k* common to the group. Asterisks following $10|F_o|$ mark reflections omitted from refinements.
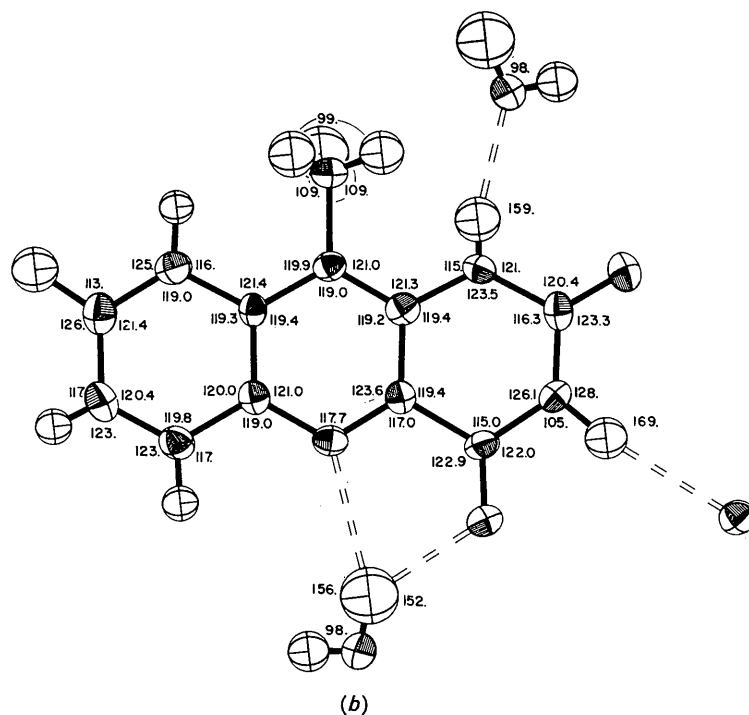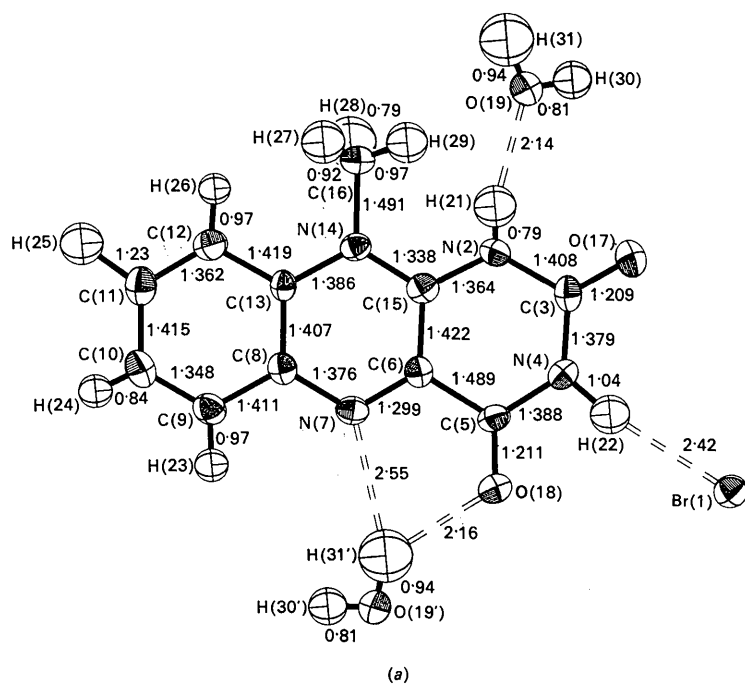
(a)

(b)

Fig. 1. (a) Interatomic distances. The standard deviation is about 0·007 Å for bonds between heavy atoms and 0·05 Å for bonds involving hydrogen. (b) Bond angle standard deviations are about 0·8° for angles involving only heavy atoms, and 4° for those involving hydrogen atoms. H(27)–C(16)–H(28) is 114°, H(28)–C(16)–H(29) is 112°, and H(28)–C(16)–N(14) is 112°.

factors gave $R = 5\cdot6\%$. Isotropic refinement of the hydrogen atoms then produced an R of $5\cdot3\%$. The remaining two water hydrogen atoms were located on a difference map, the imaginary component of the bromine anomalous scattering factor was included in refinement, and a final R of $4\cdot8\%$ was obtained.

Scattering factors were those in *International Tables for X-ray Crystallography* (1962) except for hydrogen (Stewart, Davidson & Simpson, 1965). Computations were performed on the Tulane Computer Laboratory IBM 7044 computer with programs: *BLSA* for least-squares, modified from the block-diagonal form of *UCLALS* 1 (Gantzel, Sparks & Trueblood, 1962); *FOUR* for Fourier summation (C.J.Fritchie, Jr); *GSET*4 for goniometer orientation (C.T.Prewitt); *ORTEP* for plotting figures (C.Johnson); and *CELL* for determining unit-cell constants (B.L.Trus).

The final positional parameters appear in Table 1 and the thermal parameters in Table 2. Observed structure factors are given in Table 3. Of the unobserved structure factors, 40 exceed the observational threshold by amounts less than σ_F, twelve have errors in the range $\sigma - 2\sigma$, one in the range $2\sigma - 3\sigma$, and two have larger relative errors. These latter ($10\overline{2}$ with $F_o \le 4\cdot3$, $F_c = -10\cdot1$ and $\sigma_F = 0\cdot90$; and 020 with $F_o \le 3\cdot8$, $F_c = -13\cdot8$ and $\sigma_F = 1\cdot03$) are believed to have standard deviations, and probably minimum observable magnitudes, which are seriously underestimated.

Discussion

Molecular configuration

Dudley *et al.* (Dudley, Ehrenberg, Hemmerich & Müller, 1964) have deduced, on spectroscopic grounds, that protonation of isoalloxazine (flavin) occurs at N[1]. N[1] is in fact the site of protonation in riboflavin hydrobromide hydrate (Tanaka, Ashida, Sasada & Kakudo, 1967, hereafter TASK), and also in the present structure. Both of these structures also confirm that the generally accepted diketo tautomer is the correct one.

The bond lengths given by TASK agree with ours [Fig.1(*a*)] within 2σ (TASK $\sigma_{C-C} = 0\cdot02$ Å) except for C(10)–C(11), for which they report a length of $1\cdot47$ Å. Even this is only $2\cdot5\sigma$ from our value of $1\cdot415$ Å. Their angles also agree with ours within 3σ. Our values appear in Fig.1(*b*). Surprisingly, bond lengths in 1,3,10-trimethylisoalloxazinium iodide (Kierkegaard, Norrestam, Werner, Ehrenberg, Eriksson & Müller, 1967) differ by $0\cdot04$–$0\cdot10$ Å from ours in several instances, despite their low R value ($5\cdot4\%$, σ unstated). The disagreement is probably a result of the heavy iodine atom in the trimethylisoalloxazinium salt. Supported by the work of TASK, we consider our parameters accurate to within the standard deviations given by the least-squares analysis ($\sigma_{C-C} = 0\cdot007$ Å and $\sigma_{C-C-C} = 0\cdot8\,^{\circ}$). The bond lengths in the CO–NH–CO region of the molecule are in good agreement with values in several similar molecules, as is

shown in Table 4. Similarly, the generally large intracyclic angles at nitrogen atoms in the outer ring are a common feature of such systems (Singh, 1965*a*, 1965*b*; Reeke & Marsh, 1966).

Table 4. *Comparison with bond lengths in similar molecules*

Bond	This work	RM	S
C(3)–O(17)	1·209 Å	1·229 Å	1·208 Å
			1·209
C(3)–N(4)	1·379	1·363	1·370
			1·379
N(4)–C(5)	1·388	1·381	1·374
			1·372
C(5)–O(18)	1·211	1·230	1·221

RM is 5-ethyl-6-methyluracil, Reeke & Marsh (1966). ($\sigma_{bond} = 0\cdot005$ Å); S is alloxan (hydrated), Singh (1965*a*). ($\sigma_{bond} = 0\cdot005$ Å).

Isoalloxazine bond lengths, calculated from the results of Pariser–Parr–Pople self-consistent-field molecular orbital calculations (Fox, Nishimoto & Forster, 1965) by use of the Nishimoto–Forster (Nishimoto & Forster, 1966) approximations, are compared with the observed values in Table 5. The agreement in the phenylene ring is particularly good. The prediction that C(9)–C(10) and C(11)–C(12) are shorter and other bonds longer than the benzenoid value of $1\cdot396$ Å is verified. Comparison beyond this point becomes uncertain since the calculations were performed for neutral, rather than N[1] protonated, flavin. However, bonds N(7)–C(8) and C(13)–N(14) are expected to change but little upon protonation, as is the CO–NH–CO grouping. The observed and calculated values here are in reasonable agreement. considering the predilection of the method for predicting long C–O bonds (Fox, Laberge, Nishimoto & Forster, 1967).

Table 5. *Comparison of calculated with observed distances*

Bond	Calculated*	Observed
N(2)—C(3)	1·379 Å	1·408 Å
C(3)—O(17)	1·271	1·209
C(3)—N(4)	1·379	1·379
N(4)—C(5)	1·366	1·388
C(5)—O(18)	1·268	1·211
C(5)—C(6)	1·466	1·489
C(6)—N(7)	1·308	1·299
N(7)—C(8)	1·368	1·376
C(8)—C(9)	1·415	1·411
C(9)—C(10)	1·387	1·348
C(10)–C(11)	1·407	1·415
C(11)–C(12)	1·389	1·362
C(12)–C(13)	1·411	1·419
C(13)–N(14)	1·379	1·386
N(14)–C(15)	1·363	1·338
C(15)–N(2)	1·323	1·364
C(15)–C(6)	1·450	1·422
C(13)–C(8)	1·414	1·407

* Fox, Nishimoto & Forster, 1965. Comparison is not exact, because the calculation was performed for the nonprotonated species (see text).

It is interesting that the quasi double nature of C(6)–N(7) and quasi-single natures of C(5)–C(6) and N(2)–C(3) which are predicted by the simplest resonance forms are in fact found. The fact that N(14)–C(15) is shorter than N(2)–C(15) probably implies a greater (positive) charge at N(14), a prediction in accord with the general behavior of carbonium ions.

The molecule is nearly planar, as shown by the least-squares plane results in Table 6. It is, however, bowed to a significant degree along its long axis. Similar calculations of planes through each of the three rings separately showed that the distortion is fairly uniform over the molecule, the average deviation from each of the planes being approximately 0·01 Å. The methyl carbon atom shows no larger deviation from the plane of the central ring than do the ring atoms themselves. The two oxygen atoms are somewhat further from the least-squares plane of the end ring, about 0·03 Å.

Packing

Because of the interest in understanding the flavin-enzyme and possible flavin-substrate binding in the course of flavo-protein redox reactions and the difficulty in obtaining simple metal complexes of flavins (Hemmerich, Müller & Ehrenberg, 1965), it is important to make whatever deductions possible regarding the relative basicities of the various nitrogen and oxygen atoms. Fortunately rather extensive hydrogen bonding exists in this crystal, and some comparisons are thus possible. At the same time, any deductions made on the basis of hydrogen bonding schemes must be treated with care, since under various conditions differing schemes may prevail (Hoogsteen, 1963; Haschemeyer & Sobell, 1965).

The part of the hydrogen bonding system relevant to a discussion of the properties of 10-methylisoalloxazine is given in Fig. 1(*a*) and (*b*). Atoms O(19) and

Table 6. *Least-squares plane*

	Deviation		Deviation
N(2)	0·012 Å	N(14)	−0·082 Å
C(3)	0·044	C(15)	−0·042
N(4)	0·069	C(16)	−0·087
C(5)	−0·016	O(17)	0·057
C(6)	−0·039	O(18)	−0·032
N(7)	−0·042	H(21)	0·006
C(8)	−0·030	H(22)	−0·042
C(9)	−0·004	H(23)	−0·078
C(10)	0·057	H(24)	0·028
C(11)	0·108	H(25)	0·178
C(12)	0·035	H(26)	0·069
C(13)	−0·029		

The plane is given by the equation $0 \cdot 68260m - 0 \cdot 01943n - 0 \cdot 73053p = -1 \cdot 8435$, where m, n and p are parameters measured along orthonormal axes $m \| b \times c^*$, $n \| b$ and $p \| c^*$. Only non-hydrogen atoms were used in calculating the plane, and each was given its atomic number as weight. The method of Blow (1960) was used in calculating the plane.

Br(1) serve as receptors for hydrogen bonds from N(2)–H(21) and N(4)–H(22) respectively. The N–H$\cdots$O bond is characterized by H(21)$\cdots$O(19) = 2·14 Å, N(2)$\cdots$O(19) = 2·90 Å, and < NHO = 159°. The more reliable heavy atom separation is intermediate in the range of about 2·87–3·04 Å given in a recent tabulation by Hamilton & Ibers (1968). In the case of the NH$\cdots$Br bond, N(4)$\cdots$Br(1) is 3·44 Å, H(22)$\cdots$Br(1) is 2·42 Å, and < NHBr is 169°. The H$\cdots$Br distance is 0·7 Å, smaller than the sum of van der Waals radii (3·15 Å; Pauling, 1948) and must thus be considered a bond. The N$\cdots$Br distance of 3·44 Å is only slightly longer than distances proposed as hydrogen bonds in a number of recent structures (3·36, 3·32 Å, Subramanian, 1967; 3·35 Å, Kadoya, Hanazaki & Iitaka, 1966; 3·40 Å (bifurcated), Przybylska, 1965).

Of the three possible hydrogen-bond receptor sites, O(17), O(18), and N(7), only O(18) actually partici-
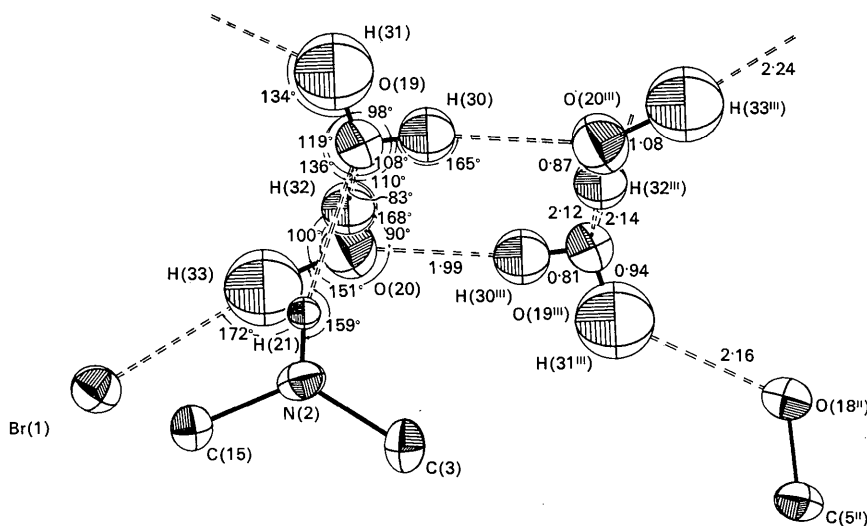

Fig. 2. A portion of the hydrogen bonding system. O(19''')$\cdots$O(20''') is 2·98 Å, O(18'')$\cdots$O(19''') is 2·89 Å, and O(19''')$\cdots$O(20) is 2·78 Å.

pates in bonding, to H(31') of a water molecule, O(18)···H(31') is 2·16 Å, O(18)···O(19') is 2·89 Å. and angle OHO is 152°. The same H(31') approaches

N(7) within 2·55 Å. This distance, only 0·15 Å less than the sum of van der Waals radii (Pauling, 1948) is not considered to be a hydrogen bond but may be
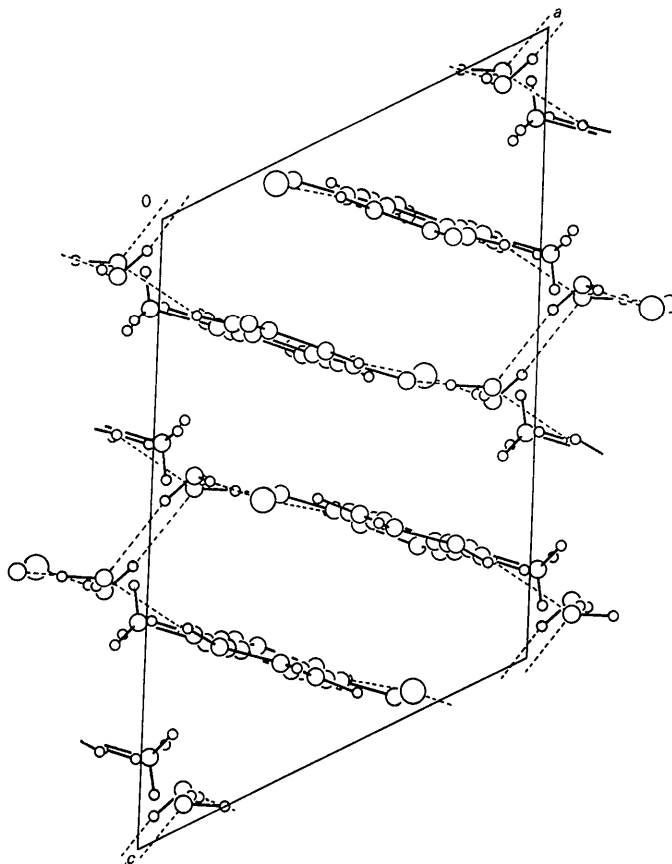

Fig. 3. [010] projection. Some of the hydrogen bonds shown are to molecules in front of, or behind, those pictured.
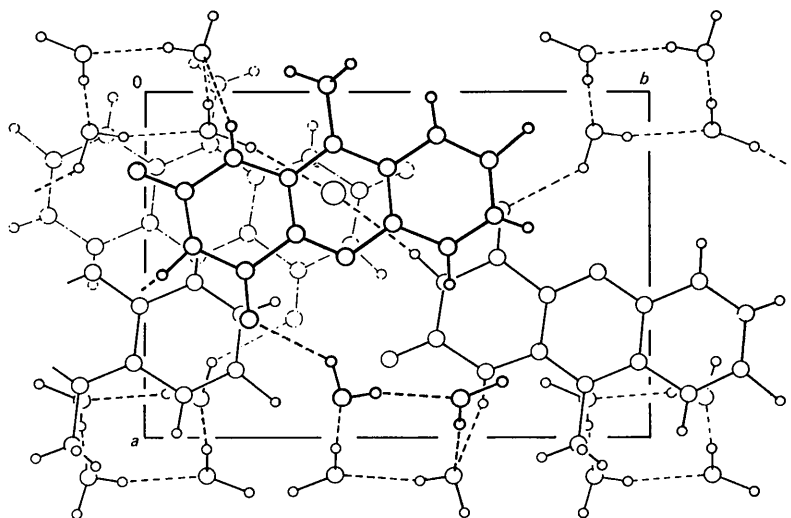

Fig. 4. [001] projection. Some of the hydrogen bonds shown involve molecules in front of, or behind, those pictured.

a stabilizing van der Waals contact. The orientation of the water molecule H(30')–O(19')–H(31') is probably determined chiefly by a hydrogen bond to O(20), shown in Fig. 2. Thus what first appears as an attempt by the structure to form a bifurcated bond from H(31') to both O(18) and N(7) is probably artefactual. We can conclude, with the reservations given above, that O(18) is the most basic of the three receptor atoms in protonated flavin.

The remainder of the hydrogen-bonding system is shown in Fig. 2 and two projections, Figs. 3 and 4. Water molecule H(32)–O(20)–H(33) is not bound to flavin, but to two other water molecules and to the bromide ion. O(19) is in a roughly tetrahedral environment, serving as a double hydrogen-bond donor and as a double acceptor. O(20) is a double donor and single receptor, and is in a pyramidal environment.

As Fig. 3 illustrates, all components of the structure lie in layers more or less parallel with (204). These layers contain some of the hydrogen bonds, with hydrogen bonds between water molecules serving to link the layers pairwise. Fig. 4 illustrates more clearly the intralayer hydrogen bonding. Flavin molecules within a layer are linked throughout by hydrogen bonds involving both the bromide ion and water molecules.

There is little direct interaction between flavin molecules, as can be seen in Fig. 4. One layer is shown in a fairly complete fashion with a single flavin molecule above and one below this sheet. The upper molecule exhibits flavin–flavin contact through C(10)–O(18) and H(23)–N(4). The only contact with the lower molecule is H(23)–N(7). The bromide ion, in addition to being a double hydrogen bond acceptor, lies fairly close to the presumably most positive areas of the flavin molecules above and below it. Thus, it is near N(14) of the upper molecule and N(2) of the lower molecule.

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