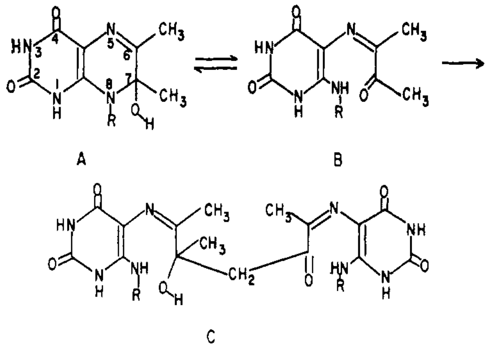
Investigations of Structures of Substituted Lumazines by Deuterium Exchange and Nuclear Magnetic Resonance Spectroscopy*

R. L. Beach† and G. W. E. Plaut

ABSTRACT: Chemical deuterium–hydrogen exchange with solvent D_2O or H_2O occurs at the substituent of carbon 7, but not at carbon 6, of several lumazines. The rate of this exchange is enhanced when a substituent is present at position 8 of the lumazine molecule and occurs most rapidly under either alkaline or acid conditions. Opening of the pyrazine ring has been demonstrated with 6,7,8-trimethyllumazine and 6,7-dimethyl-8-(1′-D-ribityl)lumazine by the changes in nuclear magnetic resonance spectra developing in alkaline solutions containing D_2O or water. The reaction is reversed by acidification. Ring opening has not been observed with lumazines unsubstituted at position 8; nevertheless, hydrogen–deuterium exchange in acid, neutral, or basic media occurred with all 7-alkyllumazine derivatives. This suggests that ring opening is not a prerequisite for the exchange reaction. Mechanisms explaining these observations have been proposed.

The exchange reaction has been used for the synthesis of 6-methyl-7-deuteriomethyl-8-(1′-D-ribityl)lumazine from the unlabeled substance and D_2O, and of 6-deuteriomethyl-7-methyl-8-(1′-D-ribityl)lumazine from 6,7-dideuteriomethyl-8-(1′-D-ribityl)lumazine and H_2O. A combination of deuterium–hydrogen exchange and nuclear magnetic resonance spectroscopy permits an unequivocal assignment of location of substituents at positions 6 and 7 of unsymmetrical 6,7-disubstituted lumazines. The utility of this technique has been illustrated here by establishing the isomeric structure of 6-ethyl-7-methyl-8-(1′-D-ribityl)lumazine.

Rowan and Wood (1963, 1968) demonstrated chemical formation of riboflavin by heating 6,7-dimethyl-8-(1′-D-ribityl)lumazine in phosphate buffer at pH 7.2. They proposed that the transformation proceeds by initial formation of a covalent hydrate of the lumazine (A), followed by reversible ring opening of the pyrazine ring (B). The ring-opened form of the lumazine is thought to form a dimer (C) by an aldol condensation mechanism. Intermediate C then cyclizes yielding 4-(1′-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine and riboflavin.



Rowan and Wood (1968) have suggested that this base-initiated mechanism may also explain the enzyme-catalyzed formation of riboflavin from 6,7-dimethyl-8-(1′-D-ribityl)lumazine (Maley and Plaut, 1959; Plaut, 1960; Wacker *et al.*, 1964). However, indications that alternate pathways for formation of flavin from the lumazine may exist have come from studies with purified enzyme (Harvey and Plaut, 1966) and the recent finding that nonenzymatic synthesis of flavin in good yield also occurs in acid media (Beach and Plaut, 1969).

A mechanism has been proposed (Beach and Plaut, 1969) in which the initial step under acid conditions involves the reversible removal of a proton from the 7-methyl group of 6,7-dimethyl-8-(1′-D-ribityl)lumazine with formation of a methenyl group at position 7. The same intermediate has been suggested previously to account for the susceptibility to air oxidation of 6,7-dimethyl-8-(1′-D-ribityl)lumazine in acid solution (Asai and Kuwada, 1962).

Reversible removal of protons from the 7-methyl group should result in hydrogen–deuterium exchange at this substituent if the compound is placed in D_2O. The replacement of hydrogen by deuterium can be followed by nuclear magnetic resonance spectroscopy since the position of absorption is proportional to the magnetic moment of the nucleus. The magnetic moment, in turn, is proportional to the spin number I, which may have values of 0, $^1/_2$, 1, $^3/_2$, etc., depending on the particular nucleus. If the mass number and atomic number of a nucleus are even, the resulting spin number I will be equal to zero and not have a magnetic moment, and thus will show no absorption when placed in a magnetic field. Protium, which has both an odd mass number and odd atomic number, yields a spin number of $I = {}^1/_2$, while

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* From the Department of Biochemistry, Rutgers Medical School, Rutgers University, New Brunswick, New Jersey 08903. *Received August 1, 1969.* Supported in part by Research Grant AM 10501 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

† Recipient of a National Science Foundation Science Faculty Fellowship (06012), 1967–1968. Present address: Rider College, Lawrenceville, N. J.

deuterium, which has an even mass number and an odd atomic number, yields a spin number of $I = 1$. Their resulting magnetic moments will be different and they will therefore interact differently in the same magnetic field resulting in absorption of protium but not deuterium in the part of the nuclear magnetic resonance spectrum studied (Dyer, 1965). The replacement of hydrogen by deuterium can, therefore, be followed since the intensity due to absorption by hydrogen will decrease as it is replaced by deuterium.

Nuclear magnetic resonance spectroscopy should also make it possible to observe the hydration of lumazines (A) as well as reversible ring opening (B) in basic media since the electron densities of these forms of the molecule are different and should result in significant chemical shifts. Decreasing absorption should accompany hydrogen–deuterium exchange at the methyl group α to the carbonyl group of compound B.

It will be shown that hydrogen–deuterium exchange occurs specifically at the 7-methyl group of 6,7-dimethyl-8-(1′-D-ribityl)lumazine and its analogs. The exchange occurs more rapidly in acid and alkali than at neutrality. Evidence will be presented that the enhancement of exchange under acidic and alkaline conditions involves different molecular forms of the lumazines. Some of the reactions studied here are also catalyzed enzymically (Plaut *et al.*, 1970).

A combination of nuclear magnetic resonance spectroscopy and the specific hydrogen–deuterium exchange has also made it possible to determine unequivocally the particular isomeric forms of lumazines unsymmetrically substituted at positions 6 and 7, a problem unresolved heretofore (Birch and Moye, 1957, 1958; Davoll and Evans, 1960; Winestock and Plaut, 1961; Rowan and Wood, 1963; Wieman *et al.*, 1966). The method of structure proof has been illustrated with 6,7-(methyl,ethyl)-8-(1′-D-ribityl)lumazine.

Experimental Procedures

Materials. The following materials used for the preparation and purification of the lumazines were purchased from the suppliers indicated: 6-chloro-2,4-dimethoxypyrimidine, 4,5-diamino-2,6-dihydroxypyrimidine hydrochloride, 2-aminoethanol, and 40% pyruvic aldehyde (Aldrich Chemical Co.), 2,3-butanedione (Fisher Scientific Co.), 2,3-pentanedione (K and K Laboratories of California), 40% methylamine (Eastman Organic Chemicals), Dowex AG-1X10 (minus 400 mesh), Dowex AG-50 WX12 (200–400 mesh), and acid alumina AG-4 (100–200 mesh) (Bio-Rad). D_2O (99.7%) (Mallinckrodt) was distilled before use.

The lumazine derivatives below were prepared by published procedures. Their authenticity was confirmed by comparison of their visible and ultraviolet spectra with those given in the literature.

6-Methyllumazine (I) was prepared by the diazotization of 2-amino-4-hydroxy-6-methylpteridine[1] (Angier *et al.*, 1952): 7-methyllumazine (II) (Sharfkin, 1959); 6,7-dimethyllumazine (III) (Weijard *et al.*, 1945); 6,7,8-trimethyllumazine (IV) (Masuda, 1957); 6,7-dimethyl-8-(2′-hydroxyethyl)lumazine (V) (Winestock and Plaut, 1961); 6,7-dimethyl-8-(1′-D-ribityl)lumazine (VI) (Maley and Plaut, 1959); 6,7-(methyl,ethyl)-8-(1′-D-ribityl)lumazine (VII) (Winestock and Plaut, 1961).

Synthesis of Deuterated Compounds

6,7-Dideuteriomethyl-8-(1′-D-ribityl)lumazine (VII′). The compound was prepared by condensing deuterated diacetyl (Herr *et al.*, 1941) with 4-(1′-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine (Maley and Plaut, 1959).

Purified diacetyl (3 ml) (Walters, 1940) was suspended in 15 ml of 1.0 N D_2SO_4 in D_2O (99.7%), sealed in an ampoule, and heated at 56° for 3 days. The solution was cooled and neutralized with excess $CaCO_3$. The deuterated diacetyl was collected by passing a stream of dry nitrogen through the solution, then through a train consisting of a tube of anhydrous $CaCl_2$ (27 × 4.25 cm) followed by three cold traps chilled with Dry Ice in acetone. The deuterated diacetyl recovered (2.5 ml) was treated again with 1 N D_2SO_4 in D_2O and purified by the same procedure, yielding 1.9 ml of deuterated diacetyl.

4-(1′-D-Ribitylamino)-5-nitroso-2,6-dihydroxypyrimidine (1.76 mmoles) was taken up in 5 ml of warm D_2O, adjusted to pH 5.8 with 2.0 N KOD, and heated to 90° in the presence of 1.0 g of sodium hydrosulfite. The pale yellow solution was cooled, deuterated diacetyl (3.60 mmoles) was added, the acidity was adjusted to pH 4.6 with D_2SO_4 (pH not corrected for pD), and the solution was heated in the dark for 40 min at 76°.

The solvent was removed under reduced pressure at 40° and the residue was dissolved in a solution containing 10 ml of D_2O and 40 ml of ethanol. This solution was then placed on a column (20 × 2.5 cm) of acid alumina (AG-4, 100–200 mesh) which had been washed previously with 300 ml of absolute ethanol to remove the fines. The column was washed with 100 ml of 80% (v/v) ethanol and the compound was eluted with 200 ml of 50% (v/v) ethanol. The solvent was removed under vacuum at 40°, and the solid was taken up in 20 ml of water and treated 23 times with 20-ml portions of water saturated benzyl alcohol. The benzyl alcohol layers were combined, filtered, an equal volume of diethyl ether was added, and the solution was treated 14 times with 25-ml portions of water. Residual benzyl alcohol was removed from the combined water layer by extraction with three 30-ml portions of diethyl ether. The solution was taken to dryness under reduced pressure at 40° and the compound was crystallized from 5 ml of water yielding 70 mg of a yellow product. A second crop (316 mg) could be recovered from the mother liquor upon addition of 20 ml of ethanol.

6-Deuteriomethyl-7-methyl-8-(1′-D-ribityl)lumazine (IX). 6,7-Dideuteriomethyl-8-(1′-D-ribityl)lumazine (VIII) (100 mg) was dissolved in 50 ml of 0.1 M acetic acid and left at room temperature in the dark for 3 days. The solvent was removed under reduced pressure and the resulting yellow residue was dissolved in 4 ml of hot water. Upon cooling overnight at 5°, 20 mg of solid was obtained. An additional 63 mg of the compound was recovered when an equal volume of absolute ethanol was added to the mother liquor.

6-Methyl-7-deuteriomethyl-8-(1′-D-ribityl)lumazine (X). 6,7-Dimethyl-8-(1′-D-ribityl)lumazine (VI) (60 mg) was dissolved in 10 ml of D_2O. After 1 week at room temperature in the dark, the solvent was removed by lyophilization. The residue was treated again with D_2O and the compound was dried by lyophilization.

The absorption spectra of the deuterated derivatives of

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[1] We wish to thank Dr. R. B. Angier of Lederle Laboratories for a generous gift of 2-amino-4-hydroxy-6-methylpteridine.

6,7-dimethyl-8-(1′-D-ribityl)lumazine (VIII-X) were in agreement with the values reported in the literature for the unlabeled compound (Winestock and Plaut, 1961). Paper chromatography of the labeled compounds revealed a single fluorescent spot in each of several solvent systems with the same migration as the unlabeled substance.

7-Deuteriomethyllumazine (XI). 7-Methyllumazine (II) (30 mg) was dissolved in 5 ml of D_2O containing 0.1 M acetic acid. The solution was heated at 70° for 1 week in a sealed ampoule in the dark. A pale yellow crystalline solid was obtained by lyophilization which gave the same ultraviolet spectrum as authentic unlabeled 7-methyllumazine.

Attempted Preparation of 6-Deuteriomethyllumazine (XII). The same conditions were used as those described for the preparation of 7-deuteriomethyllumazine (XI).

Methods

Determination of Chemical Shifts. Each compound (7.5 mg) was dissolved in 0.5 ml of D_2O (99.7%) containing 0.25% DSS.[2] The solution in a nuclear magnetic resonance tube was equilibrated at the ambient temperature of the probe (41°) of a Varian Model A-60-A spectrometer for 5 min. The DSS peak was placed at 0.00 ppm and the spectrum was determined and integrated. The sample was removed from the probe and left at 23° in the dark for 24 hr and the spectrum was determined again to estimate the degree of hydrogen–deuterium exchange at the 7-methyl group (compounds I–VII) and to verify that no other spectral changes occurred. Exchange of compounds containing deuteriomethyl groups (VIII-XI) was estimated by the same general method except that H_2O instead of D_2O was the solvent. Deviations from these conditions are indicated in the text, tables and figures.

The protiums at nitrogens 3 and 8 and at alcoholic hydroxyl groups of lumazine derivatives exchange rapidly in D_2O; no absorption peaks are observable with these groups in the regions of nuclear magnetic resonance spectra examined here.

Determination of Rate of Exchange. A quotient was calculated from the areas under the absorption peaks of the 7-methyl group and of a group which did not participate in hydrogen–deuterium exchange. Comparison was made usually with the nonexchanging 6-methyl group of the lumazine; in the case of 7-methyllumazine the methyl group of acetate in the buffer served this purpose. The slope of the straight line resulting from the plot of the log of the quotient against time was determined by the method of least squares. The first-order rate constant was calculated from the slope in the usual manner.

Iodoform Reaction. The compound (15 mg) was dissolved in 1 ml of 10% NaOH and the test was carried out by the procedure of Shriner *et al.* (1965). The precipitate of iodoform formed was dissolved in benzene, the solution was dried with anhydrous sodium sulfate, and the solvent was removed by evaporation yielding iodoform as a yellow solid (mp 118–120°).

The aqueous filtrate was acidified and extracted exhaustively with carbon tetrachloride until all iodine had been removed. The water layer was heated under reflux for 15 min and taken to dryness under vacuum. A solution of the residue in 5 ml of 1% NH_4OH was placed on a column (5 × 0.7 cm) of Dowex AG1X10 (formate form). The column was washed with 50 ml of water and the 6-methyl-7-hydroxylumazine derivatives were eluted with 0.5 M formic acid.

Results

Assignment of Chemical Shifts. SUBSTITUENTS AT POSITIONS 6 AND 7. Determination of the nuclear magnetic resonance spectrum of 6,7-dimethyl-8-(1′-D-ribityl)lumazine (VI) in D_2O showed two singlets (2.67 and 2.88 ppm) and two multiplets (one at 3.65–3.96 and the other at 4.79–4.97 ppm) (Table I). Integration of the spectrum indicated that the singlets and multiplets were due to the methyl groups and the ribityl side chain, respectively. When the spectrum was reexamined after 24 hr, the singlet at 2.88 ppm had decreased in intensity whereas that at 2.67 ppm and the multiplets were unchanged, suggesting chemical exchange of solvent deuterium with hydrogen of one of the methyl groups of 6,7-dimethyl-8-(1′-D-ribityl)lumazine.

A number of analogs of lumazine containing methyl group substituents at the 6 and/or 7 position were examined to make an unambiguous assignment of the chemical shift and the group exhibiting hydrogen–deuterium exchange. The spectra of these compounds are described in Table I.

6-Methyllumazine (I) exhibits two singlets at 2.52 and 8.59 ppm, and 7-methyllumazine (II) at 2.56 and 8.18 ppm. The peaks located upfield were assigned to the methyl groups since their intensities are three times greater than those in the corresponding downfield positions. The latter is attributable to the hydrogen atoms at positions 7 and 6 of the monomethyllumazines, respectively, since 6,7-dimethyllumazine (III) shows two singlets at 2.58 and 2.63 ppm, but none downfield.

Incubation of 7-methyllumazine (II) in D_2O leads to disappearance of the peak at 2.56 ppm with time. Under the conditions shown in Table II, this reaction has a half-life of 21 hr. No change in peak height at 2.52 ppm can be observed with 6-methyllumazine (I) under identical conditions even after 2 months. This establishes that the 7-methyl group, but not the 6-methyl group, participates in hydrogen–deuterium exchange.

Incubation of 6,7-dimethyllumazine (III) in D_2O leads to a dimunution of the peak at 2.63 ppm, but not that at 2.58 ppm (Table I). In this portion of the spectrum with other 6,7-dimethyllumazine derivatives (IV–VI) the chemical shift of the 7-methyl group, which participates in hydrogen–deuterium exchange, is always at a lower field than the 6-methyl group, which does not. It should also be noted that with 8-substituted 6,7-dimethyllumazines (IV–VI) it is only the absorbance characteristic of the 7-methyl group which decreases in D_2O and not those considerably downfield, which are associated with the substituent at position 8.

Replacement of hydrogen by deuterium at the 6-methyl by chemical synthesis leads to disappearance of the peak characteristic for this group. Thus, with 6,7-dideuteriomethyl-8-(1′-D-ribityl)lumazine (VIII) and with 6-deuteriomethyl-7-methyl-8-(1′-D-ribityl)lumazine (IX) the peak at 2.67 ppm is absent. It cannot be restored even by prolonged incubation of the compounds in H_2O.

The disappearance of the peak associated with the 7-methyl group is due to replacement of hydrogen by solvent

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[2] Abbreviations used are: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; The negative sign has been deleted preceding all numerical values of the chemical shift.

TABLE I: Chemical Shifts of Lumazines Substituted at Positions 6, 7, and 8.[a]

	Substituent					
					Position 8	
Compound	Position 6		Position 7		1′-D-Ribityl[b]	
Derivatives of Lumazine	Hydrogen	Methyl	Hydrogen	Methyl	C-1′	C-(2′–5′)
(I) 6-Methyl		2.52 (3H)^{c}	8.59 (1H)^{c}			
(II) 7-Methyl	8.18 (1H)^{c}			2.56 (3H)^{c,d}		
(XI) 7-Deuteriomethyl	8.18 (1H)^{c}			*d*		
(III) 6,7-Dimethyl		2.58 (3H)^{c}		2.63 (3H)^{c,d}		
(IV) 6,7,8-Trimethyl		2.67 (3H)^{c}		2.87 (3H)^{c,e}	4.08 (3H)^{c}	
(V) 6,7-Dimethyl-8-(2′-hydroxyethyl)		2.65 (3H)^{c}		2.85 (3H)^{c,e}	4.83 (2H)^{f}	4.05 (2H)^{f}
(VI) 6,7-Dimethyl-8-(1′-D-ribityl)		2.67 (3H)^{c}		2.88 (3H)^{c,e}	4.79–4.97 (2H)^{g}	3.65–3.96 (5H)^{g}
(VIII) 6,7-Dideuteriomethyl-8-(1′-D-ribityl)		2.67^{h}		2.88^{h,e}	4.80–4.98 (2H)^{g}	3.68–4.04 (5H)^{g}
(IX) 6-Deuteriomethyl-7-methyl-8-(1′-D-ribityl)		2.67^{h}		2.88 (3H)^{c}	4.80–4.98 (2H)^{g}	3.68–4.04 (5H)^{g}
(X) 6-Methyl-7-deuteriomethyl-8-(1′-D-ribityl)		2.67 (3H)^{c}		2.88^{h,e}	4.80–4.98 (2H)^{g}	3.65–4.04 (5H)^{g}
	Ethyl Group					
	Methylene	Methyl		Methyl		
(VII) 6-Ethyl-7-methyl-8-(1′-D-ribityl)	1.95 (2H)^{i}	1.18 (3H)^{j}		2.83 (3H)^{c,e}	4.80–4.98 (2H)^{g}	3.65–4.04 (5H)^{g}

[a] Values reported as parts per million, shifted downfield from an internal standard of DSS assigned a chemical shift of 0.00 ppm. Compounds I–VII in D_2O and VIII–XI in H_2O as described under Methods. The numbers in parentheses indicate the number of hydrogens in a particular group as determined by integration. [b] Except in the cases of compounds IV and V where the substituents at position 8 are methyl and 2′-hydroxyethyl, respectively. [c] Singlet. [d] Noticeable exchange only after 72 hr. [e] Significant exchange in less than 24 hr. [f] Triplet; J = coupling constant = 5 cps. [g] Multiplet. [h] Trace absorption due to residual protium. [i] Quartet, J = 8.5 cps. [j] Triplet, J = 8.5 cps.

deuterium and not an artifact, such as oxidative removal of the substituent. Thus, the results show that the absorbance of the singlet of a number of 7-deuteriomethyl derivatives is restored to the original peak height upon incubation in H_2O, *e.g.*, 6,7-dideuteriomethyl-8-(1′-D-ribityl)lumazine (VIII), 6-methyl-7-deuteriomethyl-8-(1′-D-ribityl)lumazine (X), and 7-deuteriomethyllumazine (XI) (Tables I and II).

The presence of a substituent at position 8 has a marked effect on the rate of the hydrogen–deuterium exchange at the 7-methyl group. Thus, near neutrality in phosphate buffer the velocity of the reaction of 6,7-dimethyl-8-(1′-D-ribityl)-lumazine (VI) with D_2O or of 6-methyl-7-deuteriomethyl-8-(1′-D-ribityl)lumazine (X) with H_2O at 23° is over 100-fold faster than the corresponding reactions of 7-methyllumazine (II) and 7-deuteriomethyllumazine (XI) at 76° (Table II). The rate of exchange is also much faster in alkaline and acid solutions than under neutral conditions (Table II).

SUBSTITUENTS AT POSITION 8. Assignment of the chemical shifts for the ribityl group of 6,7-dimethyl-8-(1′-D-ribityl)lumazine became possible by examining the spectra of compounds bearing simpler substituents in position 8. For example, 6,7,8-trimethyllumazine (IV) exhibits three singlets of equal intensity. Those at 2.67 and 2.87 ppm can be assigned to the methyl groups at positions 6 and 7, whereas that at 4.08 ppm must reflect the *N*-methyl group at position 8. In 6,7-dimethyl-8-(2′-hydroxyethyl)lumazine (V) also absorption peaks are noted which are considerably downfield from the singlets associated with the 6- and 7-methyl groups (Table I). The compound (V) exhibits two triplets of equal intensity centered at 4.83 and 4.05 ppm with identical J values of 5 cps. Each of the two triplets contains two hydrogens since the integral of each triplet correspond to two-thirds of that per methyl group. The triplets arise since the magnetic field experienced by the hydrogens of each of the two CH_2 groups of the 8-(2′-hydroxyethyl) substituent influences the spin arrangement of the protons of the adjacent group, giving rise to two sets of triplets of equal J values (Dyer, 1965). The triplet centered at 4.83 ppm is assigned to the 1′-methylene group. As expected, this absorption is considerably downfield from the 8-methyl group of 6,7,8-trimethyllumazine (IV) since the replacement of a hydrogen of the *N*-methyl group by the 2′-hydroxymethyl group should be accompanied by a downfield shift for the absorption of the resulting 1′-methylene group because the hydroxymethyl group is considerably

TABLE II: Rate of Hydrogen–Deuterium Exchange at the 7-Methyl Group of Various Lumazines.

Compound	No.	Solvent System	pH	Temp, °C	First-Order Rate Constant, min^{-1}
7-Methyllumazine	II	0.1 M Potassium phosphate and 0.05 M sodium acetate in D_2O	7.2[a]	76	5.4×10^{-4}
7-Deuteriomethyllumazine	XI	0.1 M Potassium phosphate and 0.05 M sodium acetate in H_2O	7.2	76	3.0×10^{-4}
6,7-Dimethyl-8-(1′-D-ribityl)lumazine	VI	0.1 N D_2SO_4 in D_2O	0.6[a]	23	Too fast to determine
		0.1 M Potassium phosphate in D_2O	5.8[a]	23	1.6×10^{-1}
		0.1 M Potassium phosphate in D_2O	6.8[a]	23	2.9×10^{-1}
		0.1 M NaOH in D_2O	12.5[a]	23	Too fast to determine
6-Methyl-7-deuteriomethyl-8-(1′-D-ribityl)lumazine	X	0.1 M Potassium phosphate in H_2O	5.8	23	4.6×10^{-2}
		0.05 M Potassium phosphate in H_2O	6.8	23	4.6×10^{-2}

[a] pH was not corrected for pD.

more electronegative than the hydrogen it replaces. The chemical shifts are typically seen to be at lower fields as electronegativity of the groups attached increases (inductive effects) (Dyer, 1965). The triplet centered at 4.05 ppm is assigned to the hydroxymethyl group of the 2′-hydroxyethyl substituent. This chemical shift is in the normal range seen for a hydroxymethyl group; for example, the hydroxymethyl group of ethanol is located at 3.70 ppm (V-14).[3]

The ribityl side chain of 6,7-dimethyl-8-(1′-D-ribityl)luamzine (VI) gives rise to two multiplets (Table I). The multiplet between 4.79 and 4.97 ppm integrates at two-thirds the intensity of the 6-methyl group and, therefore, contains two hydrogens. Its location corresponds to that of the 1′-methylene group of 6,7-dimethyl-8-(2′-hydroxyethyl)lumazine with a chemical shift centered at 4.83 ppm. The second multiplet extends from 3.68–4.04 ppm and integrates at five-thirds the intensity of the 6-methyl group. This absorption, therefore, represents the remaining five hydrogens located at carbons 2′ to 5′ of the ribityl substituent.

Structures of Unsymmetrically Substituted Lumazines. Since hydrogen–deuterium exchange occurs only between the solvent and the substituent at position 7 of the lumazine the structures of unsymmetrically 6,7-disubstituted lumazines can be determined by a combination of the exchange reaction and nuclear magnetic resonance spectroscopy.

For example, compound VII could be either 6-methyl-7-ethyl-8-(1′-D-ribityl)lumazine or 6-ethyl-7-methyl-8-(1′-D-ribityl)lumazine (Winestock and Plaut, 1961). The nuclear magnetic resonance spectrum of the substance (Table I) exhibits two multiplets at 4.80–4.98 and 3.65–4.04 ppm which correspond to the signal of the ribityl group of 6,7-dimethyl-8-(1′-D-ribityl)lumazine (VI). A singlet is the only part of the spectrum which decreases in intensity when the compound is incubated in D_2O and the location of its chemical shift at 2.83 ppm is in excellent agreement with that of 7-methyl groups of a number of 6,7-dimethyl-8-substituted lumazines, *e.g.*, compounds IV–VI (Table I). The ethyl group of compound VII exhibits a triplet centered at 1.18 and a quartet at 1.95 ppm, each with coupling constant $J = 8.5$ cps, which can be assigned, respectively, to the methyl and methylene portions of the ethyl group. If the ethyl group of compound VII were located at position 7 one would have expected that incubation in D_2O would lead to a decrease in the intensity of absorption of the quartet centered at 1.95 ppm and conversion of the triplet at 1.18 ppm into an apparent singlet. Substitution by deuterium of the hydrogens of the methylene would still split the hydrogens of the methyl group, but would yield a J value of less than 1 cps. Compound VII, therefore, has the structure 6-ethyl-7-methyl-8-(1′-D-ribityl)lumazine.

Effect of Alkali on Lumazines. When neutral solutions of 7-methyllumazine (II) or 6,7-dimethyllumazine (III) are made basic with NaOH, small but definite shifts in nuclear magnetic resonance spectra occur with the same general pattern of absorption maintained. However, marked changes in spectra are seen when lumazines substituted in position 8 are treated in this manner (Tables I and III). The drastic spectral changes of 8-substituted lumazines in alkali doubtlessly reflect extensive chemical changes in the molecule, such as hydration and the ring opening of the pyrazine portion of the molecules described by Rowan and Wood (1968) and Pfleiderer *et al.* (1966). Assignment of chemical shifts to specific groups of the original lumazine derivatives undergoing changes in alkali became possible by observing nuclear magnetic resonance spectra in H_2O and D_2O.

6,7,8-Trimethyllumazine. In the case of 6,7,8-trimethyl-

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[3] The letter V followed by a number refers to listing in the NMR Spectra Catalog, Varian Associates, Vol. 1 and 2 (1962–1963).

TABLE III: Chemical Shifts of Lumazines in Acid and Base.[a]

Compound	Substituent at Position				
	6		7	8	
Derivatives of Lumazine	H	CH_3	CH_3	CH_3	Ribityl
			Sodium Hydroxide (0.1 M)		
(II) 7-Methyl	8.15 (1H)		2.55[b] (3H)		
(III) 6,7-Dimethyl		2.50 (3H)	2.53[b] (3H)		
(IV) 6,7,8-Trimethyl		2.10 (3H)	3.65[c] (<3H)	3.15 (3H)	
(VI) 6,7-Dimethyl-8-(1′-D-ribityl)		2.15 (3H)	1.37[c] (<3H)		3.67–3.80 (7H)
			Hydrochloric Acid (0.1 M)		
(II) 7-Methyl	8.33 (1H)		2.63[b] (3H)		
(III) 6,7-Dimethyl		2.63[b,d] (3H)	2.66[b,d] (3H)		
(IV) 6,7,8-Trimethyl		2.80 (3H)	2.96[c] (3H)	4.25 (3H)	
(VI) 6,7-Dimethyl-8-(1′-D-ribityl)		2.71 (3H)	2.91[c] (3H)		4.82–4.98 (2H), 3.60–3.91 (5H)

[a] Values as parts per million determined as in Table I. [b] In D_2O decreased absorbance could be detected only after 30 days. [c] The hydrogen–deuterium exchange in D_2O was essentially complete within 15 min. [d] Only one singlet at 2.65 ppm remains after 10 days.

TABLE IV: Products Formed in the Iodoform Reaction from 6,7,8-Trimethyllumazine and 6,7-Dimethyl-8-(1′-D-ribityl)lumazine.[a]

Compound	6,7,8-Trimethyllumazine				
	Absorption Spectra[b]		Paper Chromatography[c]		
	max (mμ)	min (mμ)	Solvent A	Solvent B	Solvent C
Isolated 6,8-dimethyl-7-hydroxylumazine	325 280	296 242	0.55	0.42	0.32
Authentic 6,8-dimethyl-7-hydroxylumazine	325 280	296 242	0.54	0.42	0.32
	6,7-Dimethyl-8-(1′-D-ribityl)lumazine				
Isolated 6-methyl-7-hydroxy-8-(1′-D-ribityl)lumazine	325 280	299 250	0.38	0.32	0.14
Authentic 6-methyl-7-hydroxy-8-(1′-D-ribityl)lumazine	325 279	300 246	0.38	0.32	0.14

[a] The procedure is described under Methods. Iodoform (mp 118–120°) was recovered from the reaction mixtures with both compounds. [b] Spectra were determined in 0.1 M HCl in a Cary Model 14 spectrophotometer. [c] Solvent A: isobutyric acid–1 M ammonia–1% EDTA (250:150:4); solvent B: 1-butanol–ethanol–H_2O (500:175:360); solvent C: 1-butanol–acetic acid–H_2O (200:30:75).

lumazine spectral changes accompaning sequential alkalinization with sodium hydroxide and neutralization with hydrochloric acid occurring in H_2O and in D_2O are shown in Figure 1A–C and Figure 1a–c, respectively. The three singlets visible in neutral solution at 2.67, 2.87, and 4.08 ppm (Figure 1A and a) are shifted to 2.10, 3.15, and 3.65 ppm upon alkalinization in H_2O (Figure 1B) whereas in D_2O only the singlets at 2.10 and 3.15 ppm are found (Figure 1b). The peak at 3.65 ppm is associated with the 7-methyl group and disappears in D_2O because of hydrogen–deuterium exchange. This is confirmed by the observation that while neutralization with hydrochloric acid leads to complete restoration of the original spectrum in H_2O (Figure 1, *cf.* A and C) the absorption of the 7-methyl group at 3.65 ppm does not reappear when the reactions are carried out in D_2O (Figure 1, *cf.* a and c). The absorption in alkali at 2.10 ppm is probably due to the 6-methyl group since it is consistent with that of the model compound 2,3-butanedione monoxime which shows

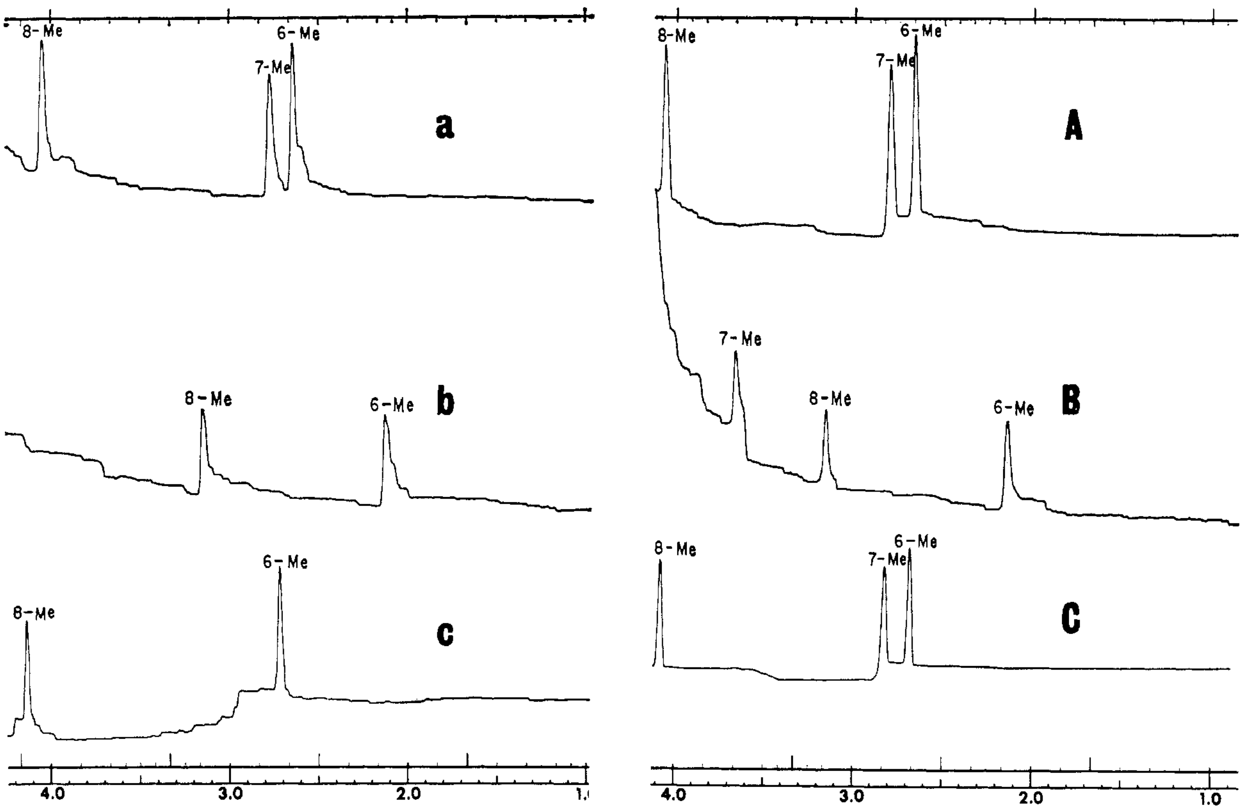

FIGURE 1: Nuclear magnetic resonance spectra of 6,7,8-trimethyllumazine. (a) A solution of the compound (7.5 mg) in 0.45 ml of D_2O, (b) alkalinized with 0.05 ml of 1.0 M NaOD, and (c) followed by neutralization with 0.05 ml of 1 M DCl. The conditions under A, B, and C were thesame as a to c, except that the solvent was H_2O. An internal standard of 0.25% DSS was present in all solutions. 6-Me, 7-Me, 8-Me, or ribityl refer to assigned positions of the 6-methyl, 7-methyl, 8-methyl, or ribityl groups, respectively, in Figures 1–3.

singlets at 2.00 and 2.40 ppm (V-72).[3] The group absorbing at 3.15 ppm (Figures 1B and b) is therefore assigned to the 8-methyl group.

6,7-Dimethyl-8-(1′-D-ribityl)lumazine. More direct evidence for the chemical shift in alkali of the 6-methyl group comes from a comparison of the spectra of 6,7-dimethyl-8-(1′-D-ribityl)lumazine (Figure 2) and 6-deuteriomethyl-7-methyl-8-(1′-D-ribityl)lumazine (Figure 3). The singlet of the 6-methyl group is present at 2.10–2.20 ppm with the unlabeled compound (Figures 2B and b), but absent when the labeled lumazine is examined (Figures 3B and b). The absorption of the corresponding 7-methyl group, a singlet at 2.88 ppm in neutral solution (Figures 2 and 3, A and a), is converted in alkaline H_2O into a multiplet centered at 1.37 ppm (Figures 2B and 3B); this is absent in D_2O due to exchange of the hydrogens with solvent deuterium (Figures 2b and 3b). Here too, neutralization of the alkaline H_2O containing solution leads to restoration of the original spectra (Figure 2 *cf.* A and C and Figure 3 *cf.* A and C) while in D_2O the peak due to the 7-methyl group disappears (Figure 2 *cf.* a and c and Figure 3 *cf.* a and c).

The multiplets assigned to the ribityl group centered at 4.90 and 3.80 ppm in neutral solution (Figures 2 and 3, a and A)[4] are shifted upfield in alkali to a single multiplet between 3.67 and 3.80 ppm (Figure 2 and 3, b and B); integration of the peak shows it to contain all 7 hydrogens of the ribityl side chain. Neutralization of the alkaline solution leads to restoration of the two multiplets in the downfield locations (Figures 2 and 3, C and c).

Iodoform Reaction. The changes in nuclear magnetic resonance spectra of 6,7-dimethyl-8-substituted lumazines in alkali would indicate a possible ring opening reaction, in which the 7-methyl group of the original lumazine is transformed to an α-methyl ketone. This is strengthened by the observation that these compounds interact rapidly with iodine in basic solution. The absorption of the 7-methyl group of 6,7,8-trimethyllumazine and 6,7-dimethyl-8-(1′-D-ribityl)lumazine at 3.65 and 1.37 ppm, respectively, disappears immediately by this treatment. The formation of iodoform has been demonstrated with both lumazine derivatives, and conversion of 6,7,8-trimethyllumazine and 6,7-dimethyl-8-(1′-D-ribityl)lumazine into 6,8-dimethyl-7-hydroxylumazine and 6-methyl-7-hydroxy-8-(1′-D-ribityl)lumazine, respectively, has been shown (Table IV).

Exchange Reaction. The hydrogen–deuterium exchange at the 7-methyl group of 8-substituted lumazines is more rapid under alkaline than neutral conditions; in 0.1 M NaOD–D_2O at 23° it is complete in less than 5 min. The rate of exchange of lumazines unsubstituted at position 8 is somewhat enhanced in alkali; however, the reaction is much slower than that with the 8-substituted compounds. For example, incubation of 7-methyllumazine or 6,7-dimethyllumazine in D_2O at

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[4] The multiplet centered at 4.90 ppm (methylene group) is barely discernable in Figures 2A and 3A since it is masked by the large absorption of the OH^- of H_2O.

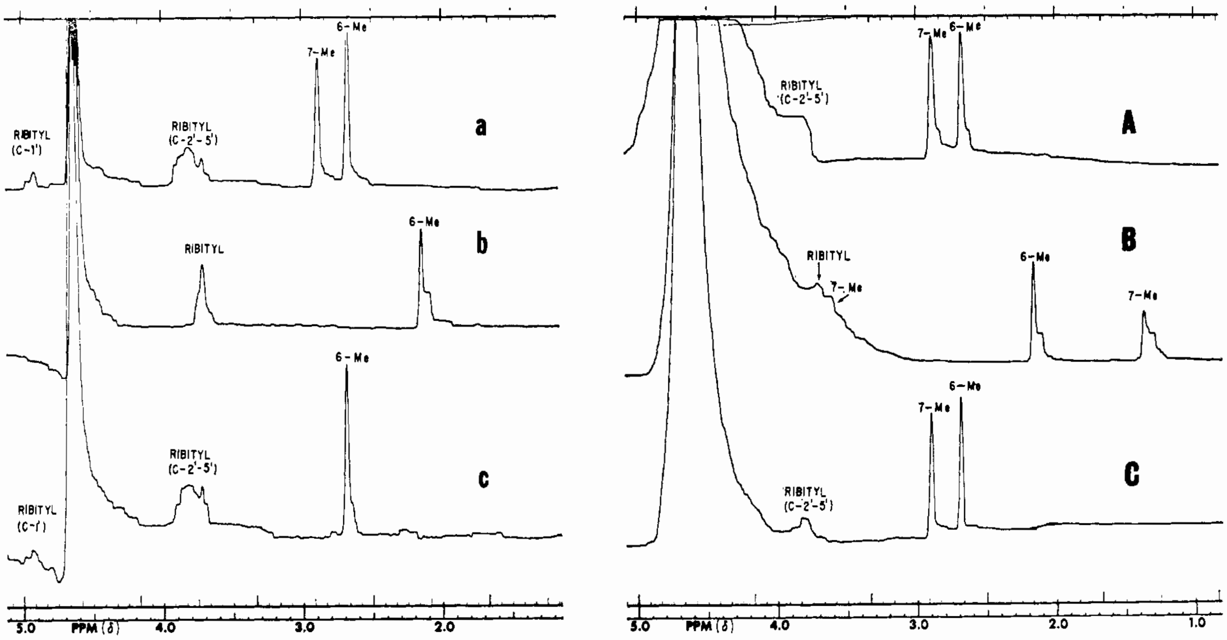


FIGURE 2: Nuclear magnetic resonance spectra of 6,7-dimethyl-8-(1′-D-ribityl)lumazine. Conditions were the same as in Figure 1.

room temperature for 30 days leads to replacement of about 70% of the hydrogen of the 7-methyl group by solvent deuterium in 0.1 M NaOD; in neutral solution less than 10% exchange occurred.

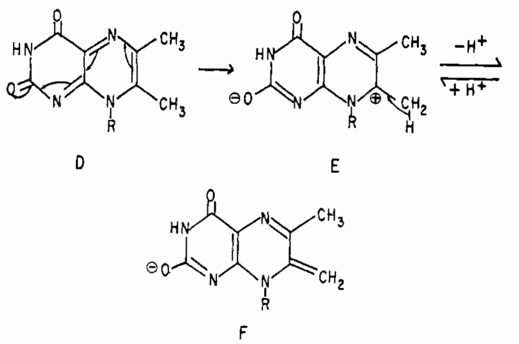
Effect of Acid on Lumazines. Acidification of neutral solutions of a number of lumazine derivatives resulted in only minor changes in nuclear magnetic resonance spectra regardless of whether or not a substituent is present at position 8 (Tables I and III), indicating that even under strongly acidic conditions (*e.g.*, 0.1 M HCl) the structures of the molecules are similar to those in neutral solution.

The rate of hydrogen–deuterium exchange at the 7-methyl group is, however, substantially different in acid and neutral solutions (Tables II and III). Acidification leads to a marked increase in exchange rates with lumazines substituted in position 8, whereas in the absence of the substituent the velocity of this reaction is depressed.

Discussion

Hydrogen–Deuterium Exchange Reaction. The observation that in neutral solution hydrogen–deuterium exchange occurs at the 7-methyl group of lumazines but not at position 6 and the significantly slower pseudo-first-order rate constants of this exchange for the deuterated compared with the protonated forms of the substrate (Table II) are consistent with the following mechanism.

It can be seen that withdrawal of electrons by either the 2-oxo or 4-oxo group generates an electrophilic center at carbon 7 of the pteridine ring (D to E); however, resonance forms cannot be written in which an electrophilic center develops at carbon 6. The stabilization of the electrophilic center at carbon 7 (E) by reversible loss of a proton from the substituent at position 7 (F) is consistent with the specificity of hydrogen–deuterium exchange.



D E F

The conversion of E into F involves the removal of a proton from the 7-methyl group and therefore should exhibit a rather large isotope effect. The isotope effects[5] of 0.55 and 0.29 for 6,7-dimethyl-8-(1′-D-ribityl)lumazine and 7-methyllumazine, respectively, determined from the rate constants (Table II), are in the range of a primary isotope effect. Such numerical values have been observed in other cases where isotopic hydrogen located at the direct reaction center is cleaved during the reaction (Melander, 1960).

Conjugation of the 7-methyl group to the electronegative 2-oxo group would also explain why the chemical shift of the 7-methyl group is always at a lower field than that of the 6-methyl group (Table I).

The slower rate of exchange of lumazines not containing an alkyl substituent at position 8 (Table II) is probably due to the lability of the nitrogen–hydrogen bond at nitrogen 8 of the pteridine ring.

This leads to utilization of the pair of electrons between nitrogen and the proton for the neutralization of the electrophilic center (G and H) rather than that derived from the

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[5] The quotient of pseudo-first-order rate constants of the exchange reactions of the 7-deuteriomethyl group with H_2O and of the 7-methyl group with D_2O.

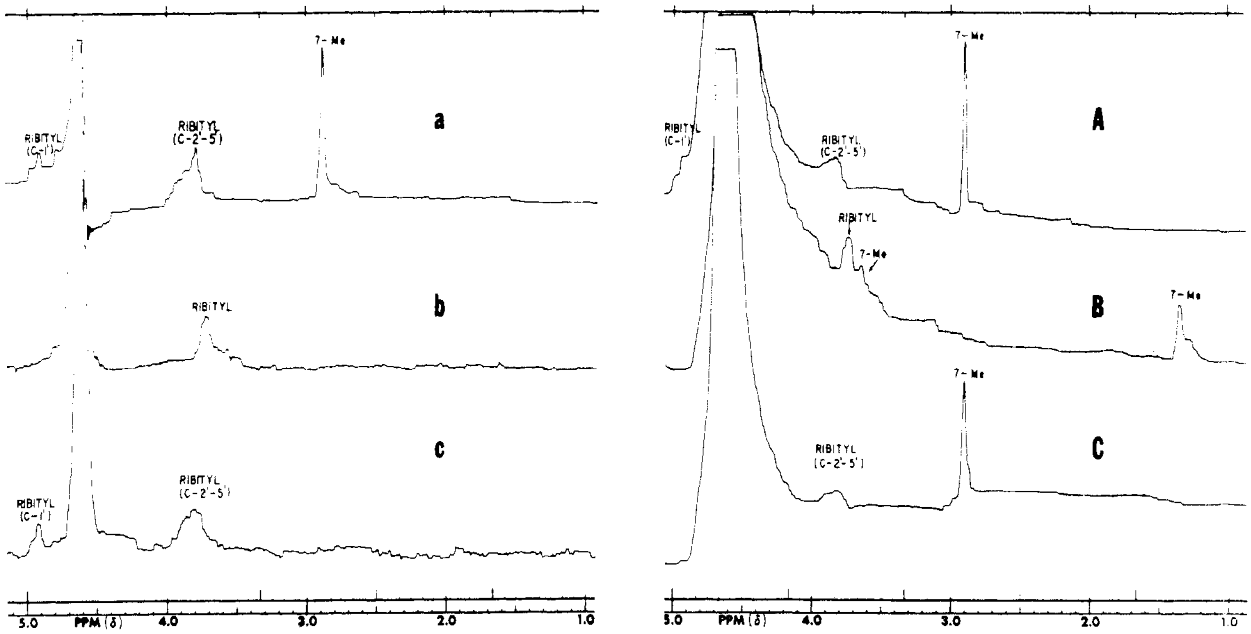
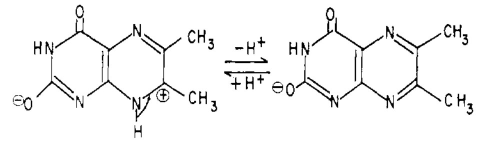


FIGURE 3: Nuclear magnetic resonance spectra of 6-deuteriomethyl-7-methyl-8-(1′-D-ribityl)lumazine. The conditions were the same as in Figure 1.

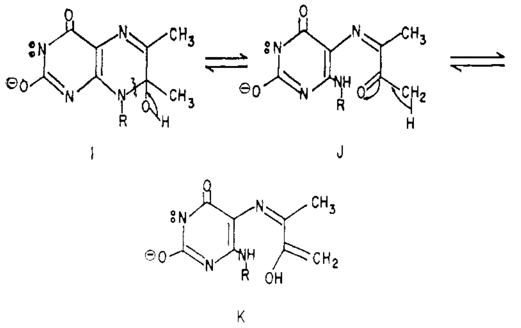


G H

7-methyl group (E and F). The latter reaction is probably obligatory for 8-substituted lumazines since under the conditions used the carbon–nitrogen bond does not cleave.

Effect of Bases. The rate of hydrogen–deuterium exchange at the 7-methyl group of lumazines is enhanced under alkaline conditions whether or not they bear a substituent at position 8. However, the presence of the substituent markedly increases the speed of the exchange. Only minor changes in nuclear magnetic resonance spectra of unsubstituted derivatives occur in base but drastic shifts suggesting marked changes in molecular structures are observed with the 8-substituted compounds (*cf.* Tables I and III).

The changes of nuclear magnetic resonance spectra of 8-substituted lumazines upon alkalinization arise either from covalent hydration of the pteridine ring and/or the subsequent opening of the pyrazine ring.



I J K

The nuclear magnetic resonance spectrum of 6,7-dimethyl-8-(1′-D-ribityl)lumazine in 0.1 M NaOH shows a major peak at 1.37 ppm and a smaller peak at 3.65 ppm, both associated with the 7-methyl group (Figures 2B and 3B). The chemical shift centered at 1.37 ppm is in the range of methylcarbinols (V-200, V-466)[3] and therefore probably reflects the hydrated form of the molecule (I). Compound I should not show hydrogen–deuterium exchange since the electrophilic center at carbon 7 would be stabilized by covalent hydration, either by an OH^- from solution or from the hydroxyl group at carbon 2′ of the ribityl side chain.[6] Since a very rapid hydrogen–deuterium exchange at the 7-methyl group does occur, the results suggest the presence of an α-methyl ketone (J) in tautomeric equilibrium with the enol form (K). The area under the peak at 1.37 ppm (7-methyl group) is only about three-fourths of that at 2.10–2.20 ppm (6-methyl group). The spectrum in 0.1 M NaOH thus appears to reflect an equilibrium mixture consisting mainly of the hydrated form (I, 1.37 ppm) and lesser amounts of the ring-opened forms (J and K, 3.65 ppm) of the molecule.

The absorption at 3.65 ppm is also observed with 6,7,8-

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[6] A form of lumazine containing an intramolecular oxygen bridge between carbons 2′ and 7, has been proposed by Pfleiderer *et al.* (1966). An attempt was made to distinguish this from the structure containing a hydroxyl group at carbon 7 (I) by comparing the nuclear magnetic resonance spectra of lumazines with and without hydroxyl groups at carbon 2′. However, both 6,7-dimethyl-8-[1′-(2′-*deoxy*-D-ribityl)]-lumazine and 6,7-dimethyl-8-(2′-*hydroxy*ethyl)lumazine exhibit singlets at 2.10 and 2.13 ppm, respectively, and negligible absorption at 1.4 and 2.20 ppm in 0.1 M NaOH (R. Beach and G. W. E. Plaut, unpublished observations). These spectra are thus similar to that of 6,7,8-trimethyllumazine (Figure 1B) which is predominantly in the ring-opened form (J) under these conditions. So far, only 6,7-dimethyl-8-(1′-D-ribityl)lumazine (Figure 2B) appears to be mainly in a form in 0.1 M NaOH which is either the hydrate (I) or which contains the proposed ether linkage between carbons 2′ and 7.

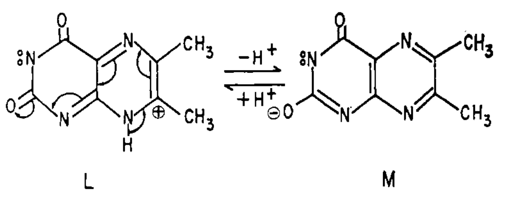
trimethyllumazine in 0.1 N NaOH where it is the only peak associated with the 7-methyl group (Figure 1B). However, the position of this resonance is considerably downfield from that generally associated with α-methyl ketone groups (2.10–2.60 ppm) (V-76, V-192),[3] the functional group of J. The absorption at 2.10 ppm of 6,7,8-trimethyllumazine and the absorption at 2.10–2.20 ppm of 6,7-dimethyl-8-(1′-D-ribityl)-lumazine (Figures 1 and 2, B and b) are associated with the 6-methyl and not the α-methyl ketone group, since the peak disappears when the 6-methyl group is in the deuterated form (Figure 3, B and b). Also, in contrast to the chemical shift at 3.65 ppm (7-methyl group), the peak at 2.10–2.20 ppm (6-methyl) does not disappear in D_2O (Figure 1B *vs.* Figure 1b, and Figure 2B *vs.* Figure 2b).

It would appear that the main difference in behavior of 6,7,8-trimethyllumazine and 6,7-dimethyl-8-(1′-D-ribityl)-lumazine in 0.1 M NaOH is the greater prevalence of the ring-opened forms (J and K) with the former and predominance of the hydrated form (I) with the latter. The absorption at 2.10 ppm is the only peak in this region with 6,7,8-trimethyllumazine (Figure 1B and 1b) and occurs with much less intensity with 6,7-dimethyl-8-(1′-D-ribityl)lumazine (Figure 2B and b); this seems to be due to the absorption of the 6-methyl group of the ring-opened form of the lumazine (J). The peak at 2.20 ppm is not seen with 6,7,8-trimethyllumazine; with 6,7-dimethyl-8-(1′-D-ribityl)lumazine it has about the same intensity as that at 1.37 ppm (7-methyl) and can be assigned to the 6-methyl group of the hydrated molecule (I).

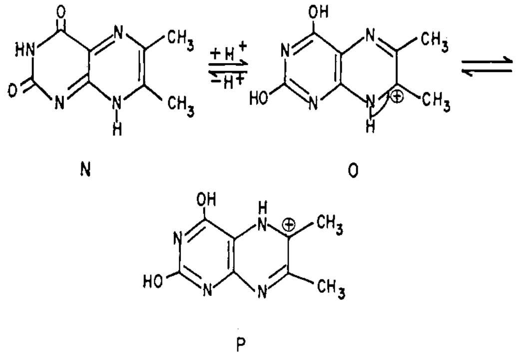
Formation in alkali of the open-ring form (J) of 6,7,8-trimethyllumazine and 6,7-dimethyl-8-(1′-D-ribityl)lumazine is supported by the additional observations that both compounds gave positive *p*-nitrophenylhydrazine (Albert *et al.*, 1962) and indoform tests. The latter also yielded the 7-oxo derivatives of the respective lumazines, the expected products of the oxidative degradation (Table IV).

The changes in nuclear magnetic resonance spectra obtained in alkali reflecting hydrated and ring-opened forms of the lumazines are reversed by neutralization (Figures 1–3). This is in agreement with the conclusion of Pfleiderer *et al.* (1966) using ultraviolet and visible light spectroscopy.

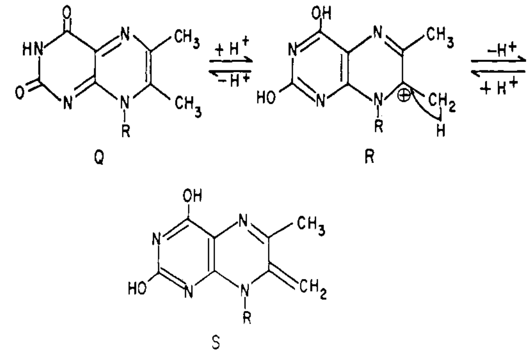
7-Methyllumazine and 6,7-dimethyllumazine do not show the changes in alkali which suggest molecular hydration or ring opening. This would suggest that the lumazines unsubstituted in position 8 act as acids in basic solution. The electrophilic center at position 7 (L) is thus stabilized by the intramolecular loss of the proton from nitrogen 8 (M), rather than by the addition of OH⁻ to form a covalent hydrate.



Effect of Acid. The nuclear magnetic resonance spectra of 6,7-dimethyllumazine are essentially the same in neutral and acid solution, regardless of whether or not a substituent is present at nitrogen 8 (Tables I and III). However, acidification markedly increases hydrogen–deuterium exchange at the 7-methyl group of 6,7-dimethyl-8-substituted lumazines (Table II); the exchange of 7-methyllumazine or 6,7-dimethyllumazine, slow at neutrality, is further depressed in acid (Tables II and III). To explain these results possible structures of these compounds as they may exist in acid solution are presented below. The neutral molecule would consist of 6- and 7-methyl groups existing in nonequivalent electronic environments (N); in acid it becomes a tautomeric mixture (O to P) in which these methyl groups become equivalent. Experimental support for this scheme comes from the observation that the compound in 0.1 M HCl shows two peaks at 2.63 and 2.66 ppm. After 10 days at 70°, only one singlet at 2.65 ppm remains; the integral of the latter single peak is essentially equal to the sum of the absorption of the singlets initially at 2.63 and 2.66 ppm. Neutralization of the electrophilic centers developed at carbons 6 and 7 is visualized to occur mainly by intramolecular migration of a proton from nitrogens 6 and 8 (O and P). As a result, hydrogen–deuterium exchange at the methyl groups would be minimal.



However, with a substituent at position 8 the tautomerism depicted above cannot occur. In the mechanism proposed below stabilization of the electrophilic center at carbon 7 occurs by reversible proton transfer from the substituent at position 7 (R and S). The enhanced hydrogen–deuterium exchange at the 7-methyl group in acid may be due to displacement of the equilibrium from Q toward R, which should be favored by the higher proton concentration.



The acceleration of hydrogen–deuterium exchange at the 7-methyl group of 8-substituted lumazines, therefore, proceeds by different mechanisms in acid and base. It is also pertinent that an initial ring-opening reaction favored in alkali, proposed as a key intermediate in chemical formation of riboflavin from 6,7-dimethyl-8-(1′-D-ribityl)lumazine under neutral conditions (Rowan and Wood, 1963, 1968), is unlikely to be a participant in the synthesis of flavin from the lumazine in acid (Beach and Plaut, 1969).[7]

Acknowledgment

We wish to express our gratitude to Rider College for the use of nuclear magnetic resonance facilities.

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[7] A mechanism of riboflavin synthesis similar to that proposed here, though differing in some details, has been proposed recently by Paterson and Wood (1969).