The Biosynthesis of Pteridines. Part VI.[1] Studies of the Mechanism of Riboflavin Biosynthesis

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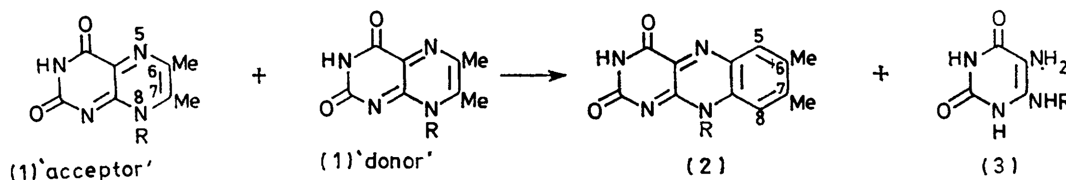
^{1}H N.m.r. spectroscopy has been used to study the deuterium exchange of the methyl protons in the lumazine precursor (6,7-dimethyl-8-D-ribitylpteridine-2,4-dione) of riboflavin, and in related compounds. The protons of the 7-methyl group exchange with the solvent, whereas those of the 6-methyl group do not. An explanation which involves a highly delocalized anionic species is suggested.

One of the labelled lumazines has been converted into the corresponding flavin by an *in vitro* reaction which closely simulates the biosynthesis of riboflavin. The distribution of deuterium label in the flavin has been established, and a mechanism for its formation is proposed. The relevance of the results to the mechanism of the enzymic synthesis of riboflavin is discussed.

THE enzyme riboflavin synthetase catalyses a unique biological reaction in which two molecules of 6,7-dimethyl-8-D-ribitylpteridine-2,4-dione (6,7-dimethyl-8-D-ribityl-lumazine) (1) form one molecule of riboflavin (2) and one molecule of 5-amino-6-D-ribitylaminouracil (3).[2-4] In this reaction a C_4 unit, derived from carbon atoms 6 and 7 and the attached methyl groups, from one molecule of the lumazine (the donor) is transferred to the second molecule of the lumazine (the acceptor) to form the *ortho*-xylene ring of riboflavin (see Scheme 1).



SCHEME 1 R = D-ribityl

The chemical synthesis[1,5] of riboflavin (2) from two molecules of 6,7-dimethyl-8-D-ribityl-lumazine (1) closely simulates the corresponding biosynthetic reaction and can be described by the same overall equation (Scheme 1). We have made use of this fact to study the conversion in detail, and we now suggest a new mechanism for the chemical reaction. Since the publication[6] of a preliminary account of our work, Beach and Plaut[7] have shown that the proposed mechanism is valid, in all essential detail, for the enzymic synthesis of riboflavin.

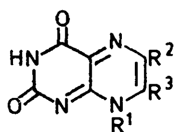
Various theories have been suggested by ourselves[1,5,8] and by others[9,10] to explain the combination of two C_4 units to give the dimethylbenzene ring of riboflavin. These proposals involve either aldol condensation of biacetyl[8,9] or a simple derivative of biacetyl,[1,5] a process which takes place around pH 13,[10] or protonation of the lumazine precursor,[11] which occurs at pH 1 or less.[12] They are thus not easily reconciled with the experimental observation that our chemical synthesis of riboflavin from the lumazine (1) occurs near neutrality, and this is presumably also the case in the enzymic synthesis. We therefore believe that these theories should be abandoned.

Deuterium Exchange of C-*Methyl Protons in* 6,7-*Dimethyl*-8-D-*ribityl-lumazine*.—Studies of the n.m.r. spectrum of the lumazine (1) in D_2O at pH 7 show that the 7-methyl protons exchange rapidly with the solvent whereas the 6-methyl protons do not (Table 1). A similar exchange is shown by other 8-substituted lumazines (4) and (5), including the 7-monomethyl derivative (6) at pH values between 7 and 13. This confirms that it is the protons of the 7-methyl group which exchange.

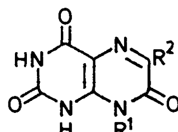
The 7-methyl-lumazine (6) used in the structural assignment was prepared by condensation of 5-amino-6-(2-hydroxyethylamino)uracil with pyruvic aldehyde. The reaction gave a single product but since the synthesis is potentially ambiguous and could lead to the 6-methyl isomer, structure (6) was confirmed by oxidative demethylation with alkaline potassium permanganate to give the known[1,13] pteridine-2,4,7-trione (7). This fact, together with an earlier report[1] that the

[1] Part V, T. Rowan and H. C. S. Wood, *J. Chem. Soc.* (*C*), 1968, 452.
[2] G. W. E. Plaut, *J. Biol. Chem.*, 1960, **235**, PC 41; 1963, **238**, 2225.
[3] H. Wacker, R. A. Harvey, C. H. Winestock, and G. W. E. Plaut, *J. Biol. Chem.*, 1964, **239**, 3493.
[4] R. A. Harvey and G. W. E. Plaut, *J. Biol. Chem.*, 1966, **241**, 2120.
[5] T. Rowan and H. C. S. Wood, *Proc. Chem. Soc.*, 1963, 21.
[6] T. Paterson and H. C. S. Wood, *Chem. Comm.*, 1969, 290.
[7] R. L. Beach and G. W. E. Plaut, *J. Amer. Chem. Soc.*, 1970, **92**, 2913.
[8] R. M. Cresswell and H. C. S. Wood, *Proc. Chem. Soc.*, 1959, 387; *J. Chem. Soc.*, 1960, 4768.
[9] A. J. Birch and C. J. Moye, *J. Chem. Soc.*, 1957, 412; 1958, 2622; A. J. Birch, *Proc. Chem. Soc.*, 1962, 3.
[10] O. Diels, W. M. Blanchard, and H. Heyden, *Ber.*, 1914, **47**, 2355.
[11] R. Beach and G. W. E. Plaut, *Tetrahedron Letters*, 1969, **40**, 3489.
[12] W. Pfleiderer, J. W. Bunting, D. D. Perrin, and G. Nübel, *Chem. Ber.*, 1966, **99**, 3503.
[13] G. Nübel and W. Pfleiderer, *Chem. Ber.*, 1962, **95**, 160.

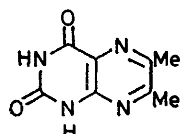
6,7-dimethyl-8-(2-hydroxyethyl)lumazine (4), on similar oxidation with permanganate, gives the 6-methylpteridine-2,4,7-trione (8) establishes that only a 7-methyl group is removed under these conditions and hence the monomethyl-lumazine has structure (6).



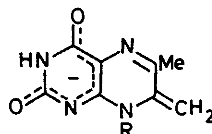
(4) $R^1 = CH_2{\cdot}CH_2OH, R^2 = R^3 = Me$
(5) $R^1 = R^2 = R^3 = Me$
(6) $R^1 = CH_2{\cdot}CH_2OH, R^2 = H, R^3 = Me$



(7) $R^1 = CH_2{\cdot}CH_2OH, R^2 = H$
(8) $R^1 = CH_2{\cdot}CH_2OH, R^2 = Me$



(9)



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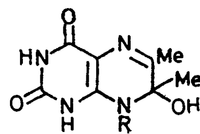
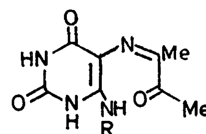
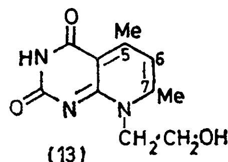


(11)



(12)



(13)

Under the conditions of our experiments we did not observe any exchange in the simple lumazine derivative (9). Since our preliminary communication,[6] two other groups[14,15] have reported in detail on the exchange phenomenon. Beach and Plaut[14] report exchange of the 7-methyl protons in the lumazine (9) but the reaction is slow (detectable exchange only after 30 days at pH 13 at room temperature). The rapid exchange is thus characteristic of the 7-methyl protons in 8-substituted lumazine derivatives, *e.g.* (1).

McAndless and Stewart[15] have studied the effect of acidity on the rate of exchange and have shown that the reaction is subject to both general acid and general base catalysis. Above pH 4 general base-catalysis becomes more important and we attribute the relative 'acidity' of the 7-methyl protons to the intermediate formation of a highly delocalized anionic species (10). Essentially similar conclusions have been reached by the other workers.[14,15]

An alternative explanation is that the exchange reaction involves successive and reversible hydration and ring-opening of the lumazine derivative [(1) ⟶ (11) ⟶ (12)] followed by enolization of the methyl ketone (12). We prefer the first explanation for the following reasons. (*a*) Riboflavin 5′-phosphate (2; R = D-ribityl 5′-phosphate) shows[16] a similar exchange of the methyl protons at position 7. No ring-opening is possible in this case. (*b*) Studies[17] of related heterocyclic systems show similar exchange phenomena. Thus the protons in both methyl groups of the 8-substituted 5,7-dimethylpyrido[2,3-*d*]pyrimidine (13) undergo exchange in D_2O at pH 13. Were ring-opening to take place in this case it would involve double fission of the ring system to give a pyrimidine and acetylacetone under conditions where these fragments do not recombine to give the pyrido[2,3-*d*]pyrimidine (13). (*c*) The mono-oxime of biacetyl, a simple analogue of the methyl ketone (12), undergoes exchange of the methyl ketone protons only slowly (85% exchange in 2·5 h) when heated in D_2O at 100°.

TABLE 1

^1H N.m.r. spectra (60 MHz; τ values)

Compound	6-Me	7-Me	Solvent
(1)	7·35	7·12	D_2O (pH *ca.* 7)
	7·35		D_2O (pH *ca.* 7) [a]
(4)	7·06	6·85	$CF_3{\cdot}CO_2H$
	7·93		NaOD (pH 13)
(5)	7·02	6·88 [b]	$CF_3{\cdot}CO_2H$
(6)		6·83	$CF_3{\cdot}CO_2H$
			NaOD (pH 13)
(9)	7·55	7·53	NaOD (pH 13)
(13)	7·00	6·91	$CF_3{\cdot}CO_2H$
			NaOD (pH 13)

[a] After 15 min at 100°. [b] Height of peak decreased by about 50% after heating in D_2O for 1 h.

Very recently, Pfleiderer, Mengel, and Hemmerich[18] have provided substantial additional evidence in favour of our suggestion.

Mechanism of the Conversion of the Lumazine Precursor into Riboflavin.—Whatever the detailed mechanism of the exchange reaction, the experiments already discussed show that the lumazine precursor (1) has some of the reactivity of a carbanionic species in essentially neutral solution.

When the deuterium-labelled lumazine (14) was refluxed in D_2O at pH 7·3 (phosphate buffer) it was converted, as in our earlier work,[1,5] into the corresponding flavin (15). The n.m.r. spectrum of the flavin (15) showed that the aromatic 8-proton and the 6-methyl protons had been replaced by deuterium (see Table 2). It was convenient to use the deuteriated 8-(2-hydroxyethyl)-6,7-dimethyl-lumazine (14) for these studies, and we have no reason to suppose that our conclusions are not valid for the natural precursor (1).

Assignment of the n.m.r. signals for the deuteriated

[14] R. L. Beach and G. W. E. Plaut, *Biochemistry*, 1970, **9**, 760
[15] J. M. McAndless and R. Stewart, *Canad. J. Chem.*, 1970, **48**, 263.
[16] F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, 1965, **30**, 2056.
[17] T. Paterson and H. C. S. Wood, preceding paper.
[18] W. Pfleiderer, R. Mengel, and P. Hemmerich, *Chem. Ber.*, 1971, **104**, 2273.

flavin (15) was based on a comparison of the spectra of lumiflavin and its 6-ethyl analogue (Table 2) in trifluoroacetic acid. This showed that the high-field

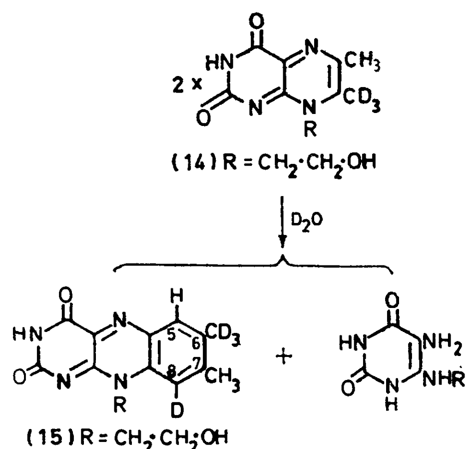
TABLE 2

^{1}H N.m.r. spectra [a] (100 MHz; τ values)

Compound	H-5	H-8	6-Me	7-Me	6-CH_2·CH_3
(2; R = CH_2·CH_2·OH)	1·66	1·77	7·32	7·20	
(15)	1·66			7·21	
(2; R = Me) (lumiflavin)	1·65	1·85	7·30	7·16	
6-Ethyl-7,9-dimethylisoalloxazine	1·66	1·88		7·17	6·98(q) 8·52(t)

[a] Solutions in CF_3·CO_2H; all signals were singlets unless otherwise stated.

signal in the methyl region is due to the 6-methyl protons, an assignment which is in agreement with that reported by Bullock and Jardetzky [16] for riboflavin 5'-phosphate in aqueous solution. Since the change of



solvent has not affected the relative positions of the signals from the methyl groups, we accept the report by Bullock and Jardetzky that the high-field signal in the aromatic region is due to the 8-proton.

The results of these experiments indicate that one of the lumazine molecules must donate a C_4 unit in some specific way to the second lumazine molecule to form the *ortho*-xylene ring of riboflavin. We suggest a mechanism (Scheme 2) for the *in vitro* reaction which is consistent with the known chemical reactivity of the lumazine precursors.

The initial step (*a*) involves removal of a proton from the 7-methyl group of one of the lumazine molecules (the acceptor), and attack by the resulting potential carbanion (16) at position 6 of a hydrated pteridine (17) formed from the second lumazine molecule (the donor) with the formation of an intermediate adduct (18). It appears to us to be essential that the second lumazine molecule reacts as a 7,8-dihydropteridine in order to explain nucleophilic attack at position 6. Were this lumazine molecule to react in the quinonoid form (1), nucleophilic attack would undoubtedly occur at position 7, and the resulting flavin would not have the labelling pattern described. The existence in neutral aqueous solution of the two reactive forms (16) and (17) of the lumazine has been demonstrated by our chemical studies reported in this and earlier papers.[1,19]

The most basic nitrogen atom in the adduct (18) is likely to be that at position 5 in the tetrahydropteridine ring. Ring-opening of the protonated form (19) of the initial adduct would lead to the simple carbinolamine (20). This would be expected to exist in equilibrium with the ketone (21) and a diaminouracil fragment, and this equilibrium might reasonably be expected to favour the ketone much more than in the case already discussed [(11) ⟶ (12)] where the carbinolamine was part of a cyclic system. Cyclisation (*c*) of the ketone (21) will give the flavin (22) with the correct distribution of deuterium label.

The attack by a nucleophilic reagent at position 6 of a 7,8-dihydropteridine finds chemical analogy in the addition reactions of 7,8-dihydropteridines to give tetrahydropteridines.[20] We have sought additional support for this hypothesis by studying nucleophilic addition to various 7,8-dihydrolumazine derivatives. Thus the oxazolo[2,3-*h*]pteridine (23), which bears a structural resemblance to the hydrated pteridine (17), was prepared by condensation of 5-amino-6-(2-hydroxyethylamino)uracil hydrochloride with bi-isobutyryl. A similar derivative (24) has been described by Pfleiderer and his co-workers.[12] We were not able to demonstrate addition of nucleophilic reagents across the 5,6-double bond of this compound, and we attribute this to a steric hindrance by the bulky isopropyl group at position 6. Evidence for steric interaction in this molecule comes from the n.m.r. spectrum, which shows that the methyl protons of the isopropyl groups are markedly non-equivalent.

Attempts to prepare the 8-substituted 'blocked' dihydropteridines (29) and (30) by reductive cyclisation of the 5-nitrouracil derivatives (26) and (27) were unsuccessful. The 5-nitrouracil (26) is readily converted into the imidazo[1,2-*c*]pyrimidine (25) in water or in dilute mineral acid (*cf.* ref. 21), and this may explain the failure to form a pteridine. We were able, however, to prepare the 'blocked' dihydropteridine (31) from the corresponding 5-nitrouracil (28). Addition of hydride ion (from sodium borohydride) took place rapidly to give the corresponding tetrahydropteridine, but we were unable to obtain evidence for the addition of carbanions across the 5,6-double bond.

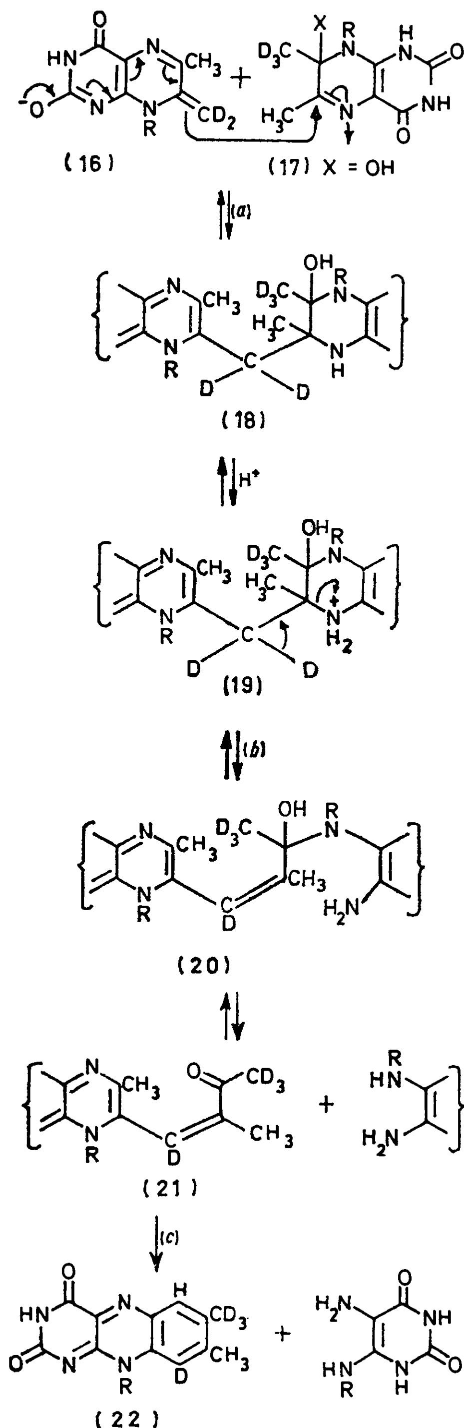
We have not therefore been able to provide direct experimental support for step (*a*) in our suggested mechanism. The results suggest that equilibria shown in Scheme 2 lie towards the right only because the final step (*c*) is irreversible and leads to formation of a stable benzene ring.

[19] T. Rowan, H. C. S. Wood, and P. Hemmerich, *Proc. Chem. Soc.*, 1961, 260.

[20] A. Stuart, H. C. S. Wood, and D. Duncan, *J. Chem. Soc.* (*C*), 1966, 285.

[21] H. Zondler and W. Pfleiderer, *Chem. Ber.*, 1966, **99**, 2984.

For the biosynthetic reaction, Beach and Plaut[7] have suggested a variation of our suggestions for the

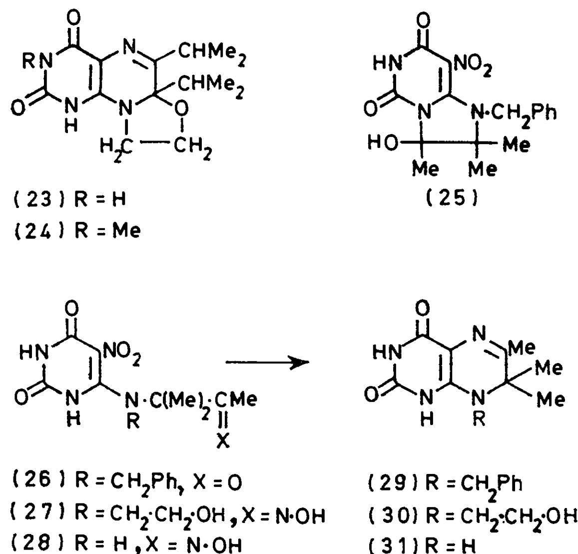


SCHEME 2

For clarity, deuterium labels are shown only on carbon atoms

final cyclisation sequence in order to avoid carbonyl intermediates such as the ketone (21). Earlier attempts[4] to trap such compounds in the enzyme-catalysed reaction were unsuccessful. It seems unlikely that this process operates in our *in vitro* reaction, and we prefer the simpler explanation given in Scheme 2.

The existence, but not the identity, of two binding sites in riboflavin synthetase has been established.[4] Our suggestions for flavin synthesis *in vitro* indicate that these sites may play a specific role in the activation of the two substrate molecules. Thus one site might



bind the acceptor lumazine molecule in such a way that a proton is readily removed to give the potential carbanion (16). A nucleophilic group such as OH or SH at the second binding site could readily react with the second lumazine molecule (the donor), converting it into a 7,8-dihydropteridine derivative [*e.g.* (17; X = *O*-enzyme or *S*-enzyme)]. Formation of riboflavin might then be expected to take place rapidly and to involve the stereospecific transfer of a C_4 unit from one lumazine molecule to the other, as suggested by our work and recently confirmed for the enzymic reaction by Beach and Plaut.[7]

EXPERIMENTAL

The purity of substances without a definite m.p. was checked by chromatography (ascending) on paper (Whatman no. 1) with (*A*) butan-1-ol–5N-acetic acid (7 : 3), (*B*) propan-1-ol–aqueous 1% ammonia (2 : 1), and (*C*) 3% ammonium chloride as solvents. Spots were located by illumination with filtered u.v. light (254 and 365 nm.), and D-ribityl derivatives were further identified by use of a periodate spray.[22]

U.v. spectra were determined with a Unicam SP 800A spectrophotometer for aqueous solutions of standard pH. N.m.r. spectra were run with either a Perkin-Elmer R10 spectrometer at 60 MHz or a Varian HA-100 instrument at 100 MHz, with tetramethylsilane as internal standard.

8-Substituted Pteridine-2,4-diones.—Most of these were prepared by published methods: 6,7-dimethyl-8-D-ribitylpteridine-2,4-dione (1);[1] 8-(2-hydroxyethyl)-6,7-dimethyl-

[22] R. L. Metzenberg and H. K. Mitchell, *J. Amer. Chem. Soc.*, 1954, **76**, 4187.

pteridine-2,4-dione (4);[1] 6,7,8-trimethylpteridine-2,4-dione (5).[8]

8-(2-Hydroxyethyl)-7-methylpteridine-2,4-dione (6).—5-Amino-6-(2-hydroxyethylamino)uracil hydrochloride[1] (500 mg, 0·0022 mol) was suspended in methanol (25 ml), aqueous 30% pyruvaldehyde solution (2·5 ml) was added, and the mixture was refluxed with stirring for 30 min. Decolourising charcoal was added and the hot solution was filtered and refrigerated. The dark green precipitate obtained gave 8-(*2-hydroxyethyl*)-7-*methylpteridine*-2,4-*dione* (255 mg, 42%) as green-yellow crystals, m.p. 287—289° (decomp.) [from aqueous ethanol (charcoal)] (Found: C, 47·6; H, 4·65; N, 24·3. $C_9H_{10}N_4O_3$, 0·25 H_2O requires C, 47·65; H, 4·65; N, 24·75%); $\lambda_{max.}$ (pH 1) 256 (log ε 4·08), 275sh (3·86), and 400 (4·01) nm; $\lambda_{max.}$ (pH 13) 241 (log ε 4·12), 265sh (3·96), 313 (4·11), and 398 (3·60) nm.

8-(2-Hydroxyethyl)pteridine-2,4,7(8H)-trione (7).—8-(2-Hydroxyethyl)-7-methylpteridine-2,4-dione (111 mg, 0·0005 mol) was dissolved in the minimum of 0·1N-sodium hydroxide and excess of 5% potassium permanganate solution was added. After 3 h at room temperature the excess of permanganate was destroyed with hydrogen peroxide and the mixture was filtered through a pad of charcoal and kieselguhr. The solution was neutralised with 0·1N-hydrochloric acid and evaporated carefully *in vacuo* to affect crystallisation. Recrystallisation from the minimum of water gave the pteridinetrione (29 mg, 26%) as pale yellow crystals, m.p. >330° (slow decomp.), u.v. spectrum identical with that of an authentic sample prepared from ethyl glyoxylate hemiacetal and 5-amino-6-(2-hydroxyethylamino)uracil hydrochloride.[1,13]

Biacetyl Mono-oxime.—The following procedure is a modification of that reported by Adams and Kamm.[23] Isopentyl nitrite (12 ml) was added dropwise, with stirring, to a mixture of ethyl methyl ketone (7·2 g, 0·1 mol), concentrated hydrochloric acid (2 ml), and ether (50 ml). During the addition the ether began to reflux gently. Aqueous 10% sodium hydrogen carbonate was added until evolution of gas had stopped. The aqueous layer was extracted with ether and the combined extracts were dried ($MgSO_4$) and evaporated *in vacuo*. The oxime (5·0 g, 50%) was obtained as crystals, m.p. 76° (from water).

Reaction of 8-(*2-Hydroxyethyl*)-6,7-*dimethylpteridine*-2,4-*dione in Deuterium Oxide Buffer* (pH 7·3).—Potassium dihydrogen phosphate was dried at 100° for several days and recrystallised from a small quantity of deuterium oxide. 2N-Sodium deuterioxide solution was added to a solution (0·1M) of the phosphate in deuterium oxide, to give a solution of pH 7·3.

The lumazine (500 mg) was refluxed in the buffer (25 ml) for 18 h under nitrogen and in the dark. On cooling, green-yellow crystals (111 mg, 36·4%) were obtained. The u.v. spectrum of the product, and its behaviour on paper chromatograms [systems (*A*)—(*C*)] were identical with those of authentic 9-(2-hydroxyethyl)-6,7-dimethylisoalloxazine.[1] The n.m.r. spectrum of the deuteriated product was obtained without further purification.

6-Ethyl-7,9-dimethylisoalloxazine.—This was prepared by condensation of 2-(*p*-carboxyphenylazo)-4-ethyl-*N*,5-dimethylaniline with barbituric acid.[16]

2,5-Dimethylhexane-3,4-dione (*Bi-isobutyryl*).—The following is based on a procedure of Wegmann and Dahn[24] for the preparation of bipropionyl. 4-Hydroxy-2,5-dimethylhexan-3-one[25] (14·4 g, 0·01 mol) and copper(II) acetate (36 g) were refluxed in aqueous 70% acetic acid (250 ml) until precipitation of red copper oxide appeared complete. Water (250 ml) was added to the pale green solution, which was then extracted with ether. The extract was washed successively with 20% sodium carbonate solution and sodium hydrogen carbonate solution, and dried (Na_2SO_4). The ether was removed *in vacuo*; fractional distillation of the residue gave bi-isobutyryl (10 g, 70%) as a pale green, pungent-smelling liquid, b.p. 63° at 30 mmHg (lit.,[26] 145—150° at 716 mmHg); $\nu_{max.}$ (film) 3426 (C=O overtone) and 1713 (C=O) cm^{-1}.

7,9,10,11-Tetrahydro-6,7-di-isopropyloxazolo[2,3-h]*pteridine*-2(1H),4(3H)-*dione* (23).—5-Amino-6-(2-hydroxyethylamino)uracil hydrochloride (2·23 g, 0·01 mol) was dissolved in water (12 ml) and bi-isobutyryl (2·0 ml) in ethanol (10 ml) was added. The mixture was refluxed for 2 h, filtered while hot, and cooled. Needles were deposited, which were purified by dissolution in 0·1N-sodium hydroxide and reprecipitation with glacial acetic acid. The *pteridine* (1·4 g, 48%) was obtained as crystals, m.p. 308—311° (decomp.) (Found: C, 57·35; H, 6·75; N, 19·1. $C_{14}H_{20}N_4O_3$ requires C, 57·55; H, 6·85; N, 19·15%); τ(NaOD) 8·73 (d), 8·92 (d), 9·12 (d), and 9·29 (d) (all CH_3·CH, *J* 7 Hz); $\lambda_{max.}$ (pH 1) 273 (log ε 4·13), 315 (3·73), and 352sh (3·32) nm; $\lambda_{max.}$ (pH 13) 231 (log ε 4·33), 282 (4·11), and 316 (3·94) nm.

Attempted nucleophilic additions were carried out as for the lumazine with similar results.

6-(N-Benzyl-1,1-dimethylacetonylamino)-5-nitrouracil (26).—2-Benzylamino-2-methylbutan-3-one[27] (0·382 g, 0·002 mol) was added to a solution of 6-chloro-5-nitrouracil (0·383 g, 0·002 mol) in ethanol (75 ml), and the mixture was left overnight at room temperature. Yellow crystals were deposited, which gave the *nitrouracil hydrochloride* (0·52 g, 68%) as a yellow powder, m.p. 166—167° (from ethanol) (Found: C, 50·35; H, 4·85; N, 14·15. $C_{16}H_{18}N_4O_5$,HCl requires C, 50·2; H, 4·95; N, 14·7%); τ(NaOD) 8·68 (6H, Me_2C), 6·44 (2H, PhCH_2), and 2·60 (5H, aromatic). Paper chromatography showed contamination by 1-benzyl-2,3-dihydro-3-hydroxy-2,2,3-trimethyl-8-nitroimidazo-[1,2-*c*]pyrimidine-5(6*H*),7(1*H*)-dione, and the n.m.r. signals (in trifluoroacetic acid) were identical with those of the imidazo[1,2-*c*]pyrimidine described later.

1-Benzyl-2,3-dihydro-3-hydroxy-2,2,3-trimethyl-8-nitro-imidazo[1,2,-c]*pyrimidine*-5(6H),8(1H)-*dione* (25).—The nitrouracil (26) hydrochloride (98 mg, 0·25 mmol) was heated at 100° in 0·1N-hydrochloric acid. Pale yellow crystals were deposited. The mixture was cooled and filtered and the solid recrystallised from water to give the *imidazopyrimidine* (53 mg, 55%) as pale yellow crystals, m.p. 252—254° (decomp.); τ(CF_3·CO_2H) 8·1 (6H, Me_2C), 7·48 (3H, CH_3·C·OH), 5·85 (2H, m, PhCH_2), and 2·47 (5H, aromatic); $\lambda_{max.}$ (pH 1) 237sh (log ε 3·72) and 318 (3·97) nm; $\lambda_{max.}$ (pH 13) 222 (log ε 4·14) and 332 (4·01) nm.

3-(2-Hydroxyethylamino)-3-methylbutan-2-one Oxime.—2-Chloro-2-methyl-3-nitrosobutane[28] (13·55 g, 0·1 mol) was added in portions to a stirred solution of ethanolamine

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(7·0 g, 0·11 mol) in ethanol (100 ml) at 0°. The mixture was allowed to warm to room temperature and was then refluxed for 8 h and set aside overnight at room temperature. The yellow solution was then evaporated *in vacuo* (20—30°) and the residue was continuously extracted with dry benzene for 6 h. The extract was concentrated *in vacuo* until crystallisation occurred. The crude product was sublimed to give the free oxime as needles (2 g, 12·4%), m.p. 84—86°.

The residue left after extraction by benzene was continuously extracted with butan-2-ol for 4 h. The solution was cooled to give the oxime hydrochloride. A further crop of hydrochloride was obtained on concentrating the mother liquor *in vacuo*. The combined *hydrochlorides* (14 g, 71%) were obtained as crystals, m.p. 177—179° (Found: C, 43·2; H, 8·85; Cl, 18·0; N, 14·45. $C_7H_{16}N_2O_2$,HCl requires C, 42·75; H, 8·65; Cl, 18·05; N, 14·25%); $\tau(D_2O)$ 8·4 (6H, Me_2C), 8·02 (3H, MeC=N), and 6·1 and 6·75 (m, $CH_2{\cdot}CH_2{\cdot}OH$); $\nu_{max.}$ (KBr) 1585 cm^{-1} (C=N).

6-[N-(2-*Hydroxyethyl*)-2-*hydroxyimino*-1,1-*dimethylpropylamino*]-5-*nitrouracil* (27).—6-Chloro-5-nitrouracil (5·75 g, 0·03 mol) and 3-(2-hydroxyethylamino)-3-methylbutan-2-one oxime hydrochloride (5·59 g, 0·03 mol) in triethylamine (9 ml) and ethanol (100 ml) were refluxed overnight. The solution was cooled and concentrated *in vacuo* until crystallisation occurred. The product was filtered off and recrystallised from ethanol to give the *nitrouracil* (7 g, 74%) as crystals (Found: C, 41·4; H, 5·2; N, 22·0. $C_{11}H_{17}N_5O_6$ requires C, 41·9; H, 5·4. N, 22·2%); $\lambda_{max.}$ (pH 1) 229 (log ε 4·37) and 324 (4·20) nm; $\lambda_{max.}$ (pH 13) 221 (log ε 4·26) and 338 (4·30) nm.

6-(2-*Hydroxyimino*-1,1-*dimethylpropylamino*)-5-*nitrouracil* (28).—6-Chloro-5-nitrouracil (4·1 g, 0·0214 mol), 3-amino-3-methylbutan-2-one oxime hydrochloride [29] (3·28 g, 0·0215 mol) and triethylamine (6·5 ml, 2 equiv.) in ethanol (75 ml) were refluxed overnight and then cooled. An off-white precipitate was obtained. The crude material was filtered off and purified by dissolving in a minimum of 2N-ammonium hydroxide and reprecipitating with glacial acetic acid. The *nitrouracil* (3·1 g, 54%) was thus obtained as a white powder, m.p. 270° (decomp.) (Found: C, 39·6; H, 4·65; N, 26·0. $C_9H_{13}N_5O_5$ requires C, 39·85; H, 4·8; N, 25·85%); τ(NaOD) 8·4 (6H, s, Me_2C) and 8·2 (3H, s, MeC=N); $\lambda_{max.}$ (pH 1) 232 (log ε 4·19) and 317 (4·14) nm; $\lambda_{max.}$ (pH 13) 223 (log ε 4·25) and 339 (4·20) nm.

7,8-*Dihydro*-6,7,7-*trimethylpteridine*-2,4-*dione* (31).—The nitrouracil (28) (1·36 g, 0·005 mol) was dissolved in the minimum of 2N-ammonium hydroxide (or 0·1N-sodium hydroxide) with gentle heating. Sodium dithionite was added in portions to the warm solution until the initial red colour had disappeared. Refrigeration of the solution gave crystals (0·7 g, 67%). Dissolution in the minimum of 2N-ammonium hydroxide and reprecipitation with glacial acetic acid gave the *pteridinedione* (0·59 g, 57%) as crystals, m.p. 300° (slow decomp.) (Found: C, 52·2; H, 6·2; N, 27·05. $C_9H_{12}N_4O_2$ requires C, 51·9; H, 5·75; N, 26·95%); $\tau(CF_3{\cdot}CO_2H)$ 8·14 (6H, Me_2C) and 7·22 (3H, MeC=N); $\lambda_{max.}$ (pH 1) 238 (log ε 4·94), 269 (4·13), and 345 (3·70) nm; $\lambda_{max.}$ (pH 13) 227 (log ε 4·29), 279 (4·08), and 315 (3·82) nm.

The dihydrolumazine (100 mg) was dissolved in the minimum of 0·1N-sodium hydroxide, and solid potassium borohydride was added in portions until no further change in the u.v. spectrum occurred. Concentrated hydrochloric acid was added and the tetrahydropteridine ($\lambda_{max.}$ 264 nm at pH 1) was obtained as needles which were rapidly reoxidised, particularly in alkaline solution, to give the dihydrolumazine.

No evidence was obtained for the similar formation of a tetrahydropteridine in the presence of a variety of other nucleophilic reagents.

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