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928. *The Biosynthesis of Pteridines. Part I. The Synthesis of Riboflavin.*

By R. M. Cresswell and H. C. S. Wood.

Condensation of 5-amino-4-D-ribitylaminouracil (II; R = D-ribityl) with the dimeric aldol (VI) from diacetyl, gives a pteridine (VII; R = D-ribityl) which readily cyclises to give riboflavin (I; R = D-ribityl). Similar condensation of a new trimeric aldol from biacetyl with the pyrimidine leads to riboflavin and 6,7-dimethyl-8-D-ribityl-lumazine (VIII; R = D-ribityl). Possible implications of these reactions in the biosynthesis of riboflavin are discussed. The synthesis of various model compounds is also described.

Recent views on the biosynthesis of pteridines suggest that they are formed from purines through an intermediate 4,5-diaminopyrimidine derivative. Albert [1] has demonstrated the conversion *in vitro* of 2-hydroxypurine into pteridine derivatives under very mild conditions, and Ziegler-Günder *et al.*[2] have shown that larvæ of the amphibian *Xenopus*

[1] Albert, *Biochem. J.*, 1957, **65**, 124.
[2] Ziegler-Günder, Simon, and Wacker, *Z. Naturforsch.*, 1956, **11**b, 82.

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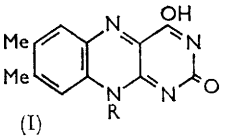
convert [2-^{14}C]guanine into a pteridine of unknown structure. Other hypotheses which have recently been reported in outline only [3,4] involve the conversion of guanosine or its 5′-phosphate into xanthopterin and related pteridines.

Many of the intermediate compounds in the above transformations are either unknown or incompletely characterised. It is our intention, in this series of papers, to describe attempts to synthesise such intermediates and to study their conversion into pteridines or related compounds.

Biochemical experiments have established that, in *Ermothecium ashbyii* and *Ashbya gossypii*, the benzo[*g*]pteridine, riboflavin (I; R = D-ribityl), is also formed from purine precursors which contribute an intact pyrimidine ring (*e.g.*, II) to the riboflavin molecule.[5,6] It has been shown in a similar manner that the dimethylbenzene ring arises from acetate units probably in the form of two molecules of biacetyl (or acetoin).[7,8] The present paper deals with the reaction between 4,5-diaminopyrimidine derivatives (II) and precursors of the benzenoid ring formed from biacetyl to give riboflavin and related isoalloxazines. A preliminary account of this work has already been published.[9]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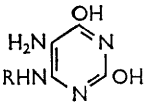
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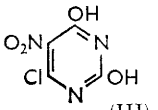
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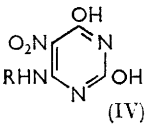
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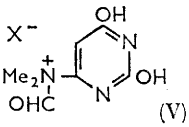
(V)

The diaminouracil derivatives (II) were prepared from 4-chlorouracil. This compound was obtained conveniently on a large scale by refluxing 2,4,6-trichloropyrimidine with 4 mol. of aqueous sodium hydroxide (we are indebted to Dr. B. W. Langley for details of this method). Nitration of 4-chlorouracil under carefully controlled conditions gave the extremely reactive 5-nitro-derivative (III). This compound with methylamine at room temperature readily gave 4-methylamino-5-nitrouracil (IV; R = Me), and similarly with ethanolamine gave the 2′-hydroxyethylamino-derivative (IV; R = CH_2·CH_2·OH). Reaction of the nitrouracil (III) with a solution of D-ribitylamine prepared by reduction of D-ribose oxime gave a crude product which was purified by chromatography on an anion-exchange resin to give 5-nitro-4-D-ribitylaminouracil (IV; R = D-ribityl). Reduction of these nitro-derivatives with sodium dithionite gave the corresponding diaminouracils (II; R = Me, CH_2·CH_2·OH, or D-ribityl) which were used directly for condensation. Attempts to isolate these compounds led to the formation of self-condensation products, the structure of which will be discussed in a subsequent paper.

5-Amino-4-2′-hydroxyethylaminouracil (II; R = CH_2·CH_2·OH) was also prepared by an alternative route which involved condensation of 4-chlorouracil with ethanolamine at 140°, followed by introduction and reduction of a nitroso-group at position 5. Attempts to apply this method to the synthesis of the ribitylamino-compound (II; R = D-ribityl) were not successful although a similar synthesis has recently been reported by Maley and Plaut.[6]

Reaction of 4-chlorouracil with ethanolamine in refluxing dimethylformamide gave

[3] Wood and Neilson, Report Brit. Emp. Cancer Campaign, 1958, **36**, 302; 1959, **37**, 649.
[4] Weygand, *Angew. Chem.*, 1959, **71**, 746.
[5] McNutt, *J. Biol. Chem.*, 1954, **210**, 511; 1956, **219**, 365; Goodwin and Pendlington, *Biochem. J.*, 1954, **57**, 631.
[6] Maley and Plaut, *J. Biol. Chem.*, 1959, **234**, 641.
[7] Birch and Moye, *J.*, 1957, 412.
[8] Plaut, *J. Biol. Chem.*, 1954, **211**, 111; Goodwin and Treble, *Biochem. J.*, 1958, **70**, 14P.
[9] Cresswell and Wood, *Proc. Chem. Soc.*, 1959, 387.

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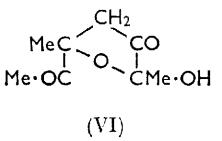
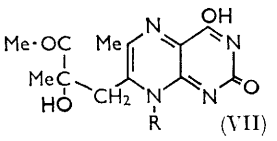
4-dimethylaminouracil and not the expected 4-2′-hydroxyethyl compound. This compound, which is also formed in somewhat lower yield by refluxing 4-chlorouracil with dimethylformamide itself, can be synthesised unambiguously by reaction of 4-chlorouracil with dimethylamine at 120°. It presumably is formed in the dimethylformamide reactions by decomposition of an intermediate quaternary salt (V).

Birch and Moye suggested [7] that the precursor of the dimethylbenzene ring of riboflavin is the aldol (VI) which is formed by self-condensation, in dilute alkali, of two molecules of biacetyl. Model experiments leading to lumichrome [7] and lumiflavin [10] (I; R = Me) support this hypothesis. We have studied the condensation of the aldol (VI) with 5-amino-4-D-ribitylaminouracil (II; R = D-ribityl) and have shown that this leads to riboflavin. The initial product of the condensation is a pteridine (VII; R = D-ribityl) (or the isomer with the substituents at positions 6 and 7 interchanged), analogous to the intermediates obtained by Birch and Moye.[7,10] The ultraviolet spectrum of this compound is almost identical with that of crystalline 6,7-dimethyl-8-D-ribityl-lumazine (VIII; R = D-ribityl) prepared by condensation of biacetyl with the pyrimidine (II; R = D-ribityl). Treatment of the pteridine (VII; R = D-ribityl) with 0·1N-sodium hydroxide or, better, with acid in the pH range 1—6 readily gave riboflavin (I; R = D-ribityl), identified by mixed melting point, paper chromatography, and ultraviolet and infrared spectra. Analogous condensations of the aldol (VI) with 5-amino-4-methylaminouracil (II; R = Me) and with the 2′-hydroxyethylamino-derivative (II; R = CH_2·CH_2·OH) gave pteridines (VII; R = Me and CH_2·CH_2·OH) which readily cyclised to lumiflavin (I; R = Me) and the 2′-hydroxyethyl analogue (I; R = CH_2·CH_2·OH).


(VI)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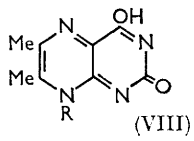
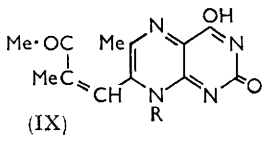

(VIII)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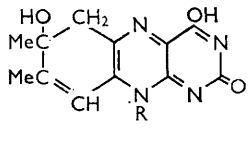
(X)

Paper chromatography of the reaction mixture from condensation of the aldol (VI) with pyrimidines (II) showed the presence, in small amount, of a second product. This has been obtained crystalline in the methyl and 2′-hydroxyethyl series and tentatively assigned structure (IX; R = Me or CH_2·CH_2OH). These compounds cannot be cyclised to the corresponding isoalloxazines (I; R = Me or CH_2·CH_2·OH) by treatment with acid or alkali and we believe this observation is more in keeping with structure (IX) than the alternative (X).

We have recently described the preparation of a new crystalline trimeric aldol of biacetyl,[9] which in certain circumstances can be formed under very mild conditions. In aqueous solution, with 4,5-diaminopyrimidine derivatives, it behaves like a mixture of biacetyl and the aldol (VI) (or the related open-chain hexanetrione). Thus, it condensed readily with 4,5-diaminouracil (II; R = H) to give a mixture of 6,7-dimethyllumazine and 2,4-dihydroxy-7-(2-hydroxy-2-methyl-3-oxobutyl)-6-methylpteridine. The latter compound is identical with material prepared [7] by reaction of the dimeric aldol (VI) with the pyrimidine (II; R = H) and on treatment with 0·1N-sodium hydroxide it gave lumichrome. Cyclisation could not be effected by treatment with acid of pH 1—6.

Similar condensation of the trimeric aldol with 5-amino-4-D-ribitylaminouracil (II; R = D-ribityl) at room temperature gave a mixture of 6,7-dimethyl-8-D-ribityl-lumazine

[10] Birch and Moye, *J.*, 1958, 2622.

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(VIII; R = D-ribityl) and the pteridine (VII; R = D-ribityl). Treatment of this mixture with 0·1N-sodium hydroxide, or with acid of pH 1—6, brought about cyclisation of the pteridine (VII; R = D-ribityl) to give crystalline riboflavin, which was readily separated from the unchanged lumazine derivative (VIII; R = D-ribityl).

Condensation of the trimeric aldol with the pyrimidines (II; R = Me and $CH_2 \cdot CH_2 \cdot OH$) gave similar results leading to lumiflavin (I; R = Me) and the 2′-hydroxyethyl analogue (I; R = $CH_2 \cdot CH_2 \cdot OH$), together with the related lumazine derivatives (VIII; R = Me and $CH_2 \cdot CH_2 \cdot OH$). In each case, the isoalloxazine separated readily in crystalline form, the more soluble lumazines being obtained by chromatography of the remaining solution.

The results obtained above, by using the dimeric aldol (VI), confirm the realistic nature of Birch's hypothesis [7] for the biosynthesis of riboflavin. Further, we believe that our results with the new trimer of biacetyl, which lead to riboflavin plus 6,7-dimethyl-8-D-ribityl-lumazine, offer a useful extension to Birch's suggestion. The following biochemical observations are all consistent with a reaction scheme such as that outlined above. 6,7-Dimethyl-8-D-ribityl-lumazine (VIII; R = D-ribityl) has been isolated, together with riboflavin (I; R = D-ribityl), from cultures of *E. ashbyii* [11] and *A. gossypii*.[6] The specific activity of the lumazine derivative (VIII; R = D-ribityl) isolated from cultures of *A. gossypii* after the addition of various radioactive precursors is approximately the same as that of riboflavin isolated in the same experiment.[6] At early stages of the incubation, however, the activity of the lumazine derivative is higher than that of riboflavin.[6] This is consistent with the latter compound's being formed in a two-stage process which does not involve the lumazine (I; R = D-ribityl).

We have also shown that the pteridines (VII; R = Me, $CH_2 \cdot CH_2 \cdot OH$, and D-ribityl) cyclise readily to isoalloxazines (I) under a variety of pH, whereas the unsubstituted analogue is cyclised to lumichrome only on treatment with 0·1N-sodium hydroxide. These experiments may explain the observation [12] that 4,5-diaminouracil (II; R = H) does not stimulate synthesis of flavins, although it is a normal metabolite of *E. ashbyii*,[13] and further biochemical work using 5-amino-4-substituted aminouracils would be of interest.

An alternative hypothesis [11,14] that 6,7-dimethyl-8-D-ribityl-lumazine (VIII; R = D-ribityl) is a precursor of riboflavin in moulds such as *E. ashbyii* and *A. gossypii* is supported by biochemical experiments using cell-free extracts of the organism. Such experiments, however, do not exclude the possibility of a second, more efficient, route for the biosynthesis of riboflavin.

Experimental

Yields of substances that have no definite m. p. refer to the stage when they appeared homogeneous in paper chromatography. Chromatograms were developed by the ascending technique, the solvents being (A) butan-1-ol–5N-acetic acid (7 : 3) and (B) 3% aqueous ammonium chloride, and were viewed in ultraviolet light of wavelengths 254 and 365 mμ. Whatman No. 1 paper was used except where stated otherwise. Infrared spectra were determined for Nujol mulls or KBr discs. R_F values and ultraviolet absorption spectra of the products obtained are given in an annexed Table.

4-*Chloro*-2,6-*dihydroxypyrimidine*.—2,4,6-Trichloropyrimidine (48 g.) was added to a solution of sodium hydroxide (41·8 g.) in water (420 c.c.) and refluxed for 16 hr. The mixture was cooled (the sodium salt of the product separating) and acidified with 12N-hydrochloric acid (50 c.c.). The precipitated product was collected, washed with water, and dried, to give 4-chlorouracil as a white powder (35·2 g., 92%), m. p. 300° (decomp.). This material is sufficiently pure for further work, but it can readily be recrystallised from boiling water (50 parts).

4-*Chloro*-2,6-*dihydroxy*-5-*nitropyrimidine* (III).—4-Chlorouracil (5 g.) was dissolved gradually

[11] Masuda, *Pharm. Bull.* (*Japan*), 1956, **4**, 375; 1957, **5**, 136.
[12] Brown, Goodwin, and Pendlington, *Biochem. J.*, 1955, **61**, 37; Korte, Aldag, and Schicke, *Z. Naturforsch.*, 1958, **13**b, 463; Korte, Aldag, Ludwig, Paulus, and Störiko, *Annalen*, 1958, **619**, 70.
[13] Goodwin and Treble, *Biochem. J.*, 1957, **67**, 10P.
[14] Maley and Plaut, *J. Amer. Chem. Soc.*, 1959, **81**, 2025.

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in 36N-sulphuric acid at <45°, and nitric acid (*d* 1·5; 5·3 c.c.) was then added slowly with stirring at 0°. The solution was kept at room temperature for 30 min., a solid separating, and then treated with ice (20 g.). The white product was collected, washed with ice-cold water (2 × 20 c.c.), and dried *in vacuo* (P_2O_5), to give the extremely reactive *4-chloro-5-nitrouracil* (2·35 g., 36%), m. p. 220—222°. Recrystallisation of this material proved impossible (Found: C, 25·8; H, 1·5. $C_4H_2ClN_3O_4$ requires C, 25·1; H, 1·1%).

Chromatographic behaviour and ultraviolet spectra of the products.

Compound	R_F in solvent (A)	R_F in solvent (B)	$\lambda_{max.}$ (mμ) (ε in parentheses [a]) in H_2O at pH given	
Pyrimidines				
4-Chlorouracil	0·67	0·67	220 (8600), 278 (10,300)	pH 13
(III)	—	—	281 (8000), 315 (8600)	pH 1
(IV; R = Me)	0·25	0·60	224 (19,000), 322 (11,600)	pH 1
			218 (15,700), 338 (14,200)	pH 13
(IV; R = CH_2·CH_2·OH)	0·25	0·64	228 (23,000), 324 (13,400)	pH 1
			216 (17,900), 333 (11,500)	pH 13
(IV; R = CH_2·CH_2·OAc)	0·38	0·70	227 (23,500), 321 (12,800)	pH 1
			218 (16,250), 337 (15,250)	pH 13
(IV; R = D-ribityl)	0·09	0·66	228 (25,700), 323 (14,200)	pH 1
			217 (17,900), 336 (17,000)	pH 13
Pteridines				
(VIII; R = CH_2·CH_2·OH)	0·19	0·76	256 (14,700), 276 [b] (10,100), 408 (11,300)	pH 1
			230 (18,600), 280 (10,700), 312 (14,200)	pH 13
(VIII; R = Me)	0·16	0·72	256 (12,200), 274 (8200), 405 (8800)	pH 1
			242 (18,100), 312 (19,600), 362 (5800)	pH 13
(VIII; R = D-ribityl)	0·10	0·78	258 (13,300), 276 [b] (8900), 408 (9700)	pH 1
			228 (15,400), 279 (11,300), 314 (7800)	pH 13
(VII; R = CH_2·CH_2·OH)	0·29	0·72	258 276 [b] 412	pH 1
(VII; R = Me)	0·24	0·62	258 276 [b] 407	pH 1
(VII; R = D-ribityl)	0·17	0·65	258 276 [b] 411	pH 1
(IX; R = CH_2·CH_2·OH)	0·40	0·56	260 (16,200), 300 [b] (9000), 430 (10,900)	pH 1
			224 (19,200), 272 (15,800), 454 (14,500)	pH 13
(IX; R = Me)	0·38	0·47	260 (21,300), 298 [b] (10,200), 434 (12,000)	pH 1
			226 (22,300), 272 (18,300), 456 (16,100)	pH 13
(IX; R = D-ribityl)	0·22	0·50	260 300 [b] 433	pH 1
			226 274 456	pH 13
Isoalloxazines				
(I; R = CH_2·CH_2·OH)	0·41	0·32	223 (31,100), 267 (29,600), 376 (9900), 444 (10,900)	pH 1
			222 (27,700), 270 (35,800), 356 (11,700), 446 (12,300)	pH 13
(I; R = Me)	0·32	0·22	222 (25,900), 265 (27,800), 375 (8800), 440 (9100)	pH 1
			220 (36,600), 269 (33,700), 353 (9100), 444 (9300)	pH 13
(I; R = D-ribityl)	0·22	0·37	223 (35,500), 267 (35,500), 376 (10,700), 445 (11,500)	pH 1
			222 (26,600), 270 (31,700), 356 (10,600), 447 (10,600)	pH 13

[a] Where no value for the extinction coefficient is given, the spectrum is that given by a single spot or band eluted from paper chromatograms. [b] Shoulder.

2,6-*Dihydroxy*-4-*methylamino*-5-*nitropyrimidine* (IV; R = Me).—4-Chloro-5-nitrouracil (1·3 g.) in ethanol (60 c.c.) was treated with methylamine (2 equiv.) in alcohol. The mixture was heated to boiling and water added dropwise to effect dissolution. On cooling, the *methylaminopyrimidine* (1·1 g., 87%) separated as needles, m. p. >300° (Found: C, 32·1; H, 3·6; N, 29·5. $C_5H_6N_4O_4$ requires C, 32·3; H, 3·3; N, 30·1%).

2,6-*Dihydroxy*-4-2′-*hydroxyethylamino*-5-*nitropyrimidine* (IV; R = CH_2·CH_2·OH).—An analogous condensation using 4-chloro-5-nitrouracil and ethanolamine gave the *2′-hydroxyethylaminopyrimidine* (62%) as needles, m. p. 217—219° (Found: C, 33·6; H, 3·8; N, 26·2. $C_6H_8N_4O_5$ requires C, 33·3; H, 3·7; N, 26·0%).

This pyrimidine with pyridine and acetic anhydride gave the *acetate* as needles, m. p. 326° (Found: C, 37·3; H, 3·5; N, 22·1. $C_8H_{10}N_4O_6$ requires C, 37·2; H, 3·9; N, 21·7%).

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D-*Ribitylamine*.—This was prepared by catalytic reduction [15] of D-ribose oxime [16] in presence of a platinum catalyst. The resulting solution, after removal of the catalyst, was shown to contain 96% of amine by titration with standard acid. Ion-exchange chromatography of a sample showed the presence of two non-volatile basic components in the solution.

2,6-*Dihydroxy*-5-*nitro*-4-D-*ribitylaminopyrimidine* (IV; R = D-ribityl).—To a solution of crude D-ribitylamine (from 6·6 g. of oxime) was added 4-chloro-5-nitrouracil (3·9 g.) in ethanol (200 c.c.). The resulting solution was left at room temperature for 24 hr. A solid (1 g.) separated and was collected and identified as 4-amino-2,6-dihydroxy-5-nitropyrimidine.[17] The pH of the filtrate was adjusted to 10·7 with ammonium formate buffer, and the solution run on to a column of the anion-exchange resin (Amberlite CG 400; formate form) which had previously been prepared by washing the resin with ammonium formate buffer (0·1M with respect to formic acid) at pH 10·7. The column was washed thoroughly with ammonium formate buffer of pH 10·7, and then with buffer of pH 7·4 (0·1M with respect to formic acid); a bright yellow pyrimidine was eluted that showed ultraviolet absorption maxima at 218 and 334 mμ at pH 13 but is as yet unidentified.

Elution with buffer of pH 4 (0·1M with respect to ammonia) eluted a second pyrimidine in a narrow band. Evaporation of this eluate at <40° to about 10 c.c., followed by the addition of ethanol (500 c.c.), gave a white solid. This was freed from ammonium formate by boiling ethanol (150 c.c.) in which ammonium formate is soluble. The resulting *ribitylaminopyrimidine* (2·7 g.), m. p. 203—204°, could not be recrystallised (Found: C, 33·8; H, 4·8; N, 17·4. $C_9H_{14}N_4O_8,H_2O$ requires C, 33·3; H, 5·0; N, 17·3%); it had $[\alpha]_D^{23}$ +4·5° (*c* 1·25 in 0·1N-NaOH).

2,6-*Dihydroxy*-4-2′-*hydroxyethylaminopyrimidine*.—4-Chlorouracil (2 g.) and ethanolamine (2 g.) in water (50 c.c.) were heated at 140° for 2 hr. in a sealed tube. Water was removed from the mixture *in vacuo* and the residue recrystallised thrice from water, to give the *pyrimidine* (0·31 g.) as needles, m. p. 256—258° (Found: C, 42·0; H, 5·2; N, 24·3. $C_6H_9N_3O_3$ requires C, 42·1; H, 5·3; N, 24·6%).

2,6-*Dihydroxy*-4-2′-*hydroxyethylamino*-5-*nitrosopyrimidine*.—2,6-Dihydroxy-4-2′-hydroxyethylaminopyrimidine (0·13 g.) was dissolved in hot 2N-hydrochloric acid (3 c.c.). To the chilled solution was added, dropwise, sodium nitrite (0·16 g.) in water (1·6 c.c.), and the solution was then allowed to come to room temperature. A pink solid (0·14 g.) that separated recrystallised (twice) from water, to give the *nitrosopyrimidine* as purple plates, m. p. 240° (decomp.) (Found: C, 36·1; H, 3·9; N, 28·0. $C_6H_8N_4O_4$ requires C, 36·0; H, 4·0; N, 28·0%).

4-*Dimethylamino*-2,6-*dihydroxypyrimidine*.—(*a*) 4-Chlorouracil (0·5 g.), dimethylamine (3 c.c. of a 33% w/w ethanolic solution), and ethanol (25 c.c.) were heated at 120° for 4 hr. in a sealed tube. The resulting solution was taken to dryness and the residue recrystallised thrice from water to give the *dimethylaminopyrimidine* as plates (0·375 g.), m. p. >300° (lit.,[18] >300°) (Found: C, 46·5; H, 5·5; N, 27·0. Calc. for $C_6H_9N_3O_2$: C, 46·4; H, 5·8; N, 27·1%).

(*b*) 4-Chlorouracil (3 g.) and ethanolamine (3 g.) were refluxed for 2 hr. in dimethylformamide (50 c.c.). The white product which separated on cooling, and a further quantity obtained by evaporation of the filtrate, were recrystallised twice from water to give the pyrimidine (0·75 g.) as plates, m. p. >300° (Found: C, 46·4; H, 5·2; N, 28·5%). The infrared spectrum of this material was identical with that prepared as in (*a*) above.

(*c*) This dimethylaminopyrimidine was also obtained by refluxing 4-chlorouracil with dimethylformamide for 2 hr.

8-2′-*Hydroxyethyl*-6,7-*dimethyl*-*lumazine* (VIII; R = CH_2·CH_2·OH).—2,6-Dihydroxy-4-2′-hydroxyethylamino-5-nitropyrimidine (0·4 g.) in water (50 c.c.) was hydrogenated over a platinum oxide (0·2 g.) catalyst. The theoretical amount of hydrogen was taken up in 5 hr. after which the hydrogen in the apparatus was replaced by nitrogen and the solution was treated with biacetyl (0·3 g.) in ethanol (25 c.c.). After 30 min. at room temperature the catalyst was removed and the green-fluorescing solution was reduced to *ca.* 10 c.c. at <40° and then refrigerated. The resulting crystals recrystallised from water to give the *pteridine* as dark orange needles (0·05 g.), m. p. 270° (decomp.) (Found: C, 51·0; H, 5·3; N, 23·8. $C_{10}H_{12}N_4O_3$ requires C, 50·8; H, 5·1; N, 23·7%).

A similar product can be obtained from the 5-nitrosopyrimidine.

[15] Kuhn, Desnuelle, and Weygand, *Ber.*, 1937, **70**, 1293.
[16] Kuhn, Reinemund, Weygand, and Ströbele, *Ber.*, 1935, **68**, 1765.
[17] Bitterli and Erlenmeyer, *Helv. Chim. Acta*, 1951, **34**, 835.
[18] King and King, *J.*, 1947 726.

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6,7,8-*Trimethyl-lumazine* (VIII; R = Me).—Reduction of 2,6-dihydroxy-4-methylamino-5-nitropyrimidine by sodium dithionite followed by condensation with biacetyl as described by Birch and Moye [10] gave the pteridine, m. p. 308—310 (lit., 320—322°).

6,7-*Dimethyl*-8-D-*ribityl-lumazine* (VIII; R = D-ribityl).—Catalytic reduction of the appropriate 5-nitropyrimidine as above, followed by condensation with biacetyl, gave the pteridine as orange needles with an absorption spectrum in agreement with that in the literature.[6]

9-2′-*Hydroxyethyl*-6,7-*dimethylisoalloxazine*.—(a) *From dimeric biacetyl* (VI). 2,6-Dihydroxy-4-2′-hydroxyethylamino-5-nitropyrimidine (0·5 g.) was dissolved in water (10 c.c.) at 85—90°. To the hot solution was added 10% aqueous sodium hydroxide (3 c.c.), followed by sodium dithionite portionwise until the colour was discharged. 12N-Hydrochloric acid was added to pH 4, followed by dimeric biacetyl (0·5 g.), and the solution was heated for 15 min. at 90°. The resulting mixture was separated by paper chromatography on several sheets of Whatman paper No. 17, with solvent system (A). Two components separated readily, and these were eluted from the dried chromatograms with water (400 c.c. in each case). The band with the lower R_F value had an ultraviolet absorption spectrum almost identical with that of the pteridine (VIII; R = CH_2·CH_2·OH) (see Table), but differed in R_F value from this compound. Evaporation of the eluate *in vacuo* to about 10 c.c. gave 8-2′-hydroxyethyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-6-methyl-lumazine (VII; R = CH_2·CH_2·OH) (or the isomer with the substituents at positions 6 and 7 interchanged) as a very hygroscopic orange solid which rapidly decomposed to a brown gum. Elementary analysis was not possible and the constitution assigned to this compound is based on its spectral characteristics, and on its very ready conversion into the isoalloxazine (I; R = CH_2·CH_2·OH) by the methods described below. The eluate, containing the compound of higher R_F value on evaporation gave 8-2′-*hydroxy*-6-*methyl*-7-(2-*methyl*-3-*oxobut*-1-*enyl*)-*lumazine* (IX; R = CH_2·CH_2·OH) as orange needles (0·025 g.), m. p. 266—268° (Found: C, 54·6; H, 5·2; N, 18·8. $C_{14}H_{16}N_4O_4$ requires C, 55·2; H, 5·3; N, 18·4%). This compound was not converted into the isoalloxazine (I; R = CH_2·CH_2·OH) by acid or alkali.

A similar reaction was carried out with 0·36 g. of the 5-nitropyrimidine, the intermediate pteridine (VII; R = CH_2·CH_2·OH) being cyclised directly to the isoalloxazine. Thus the solution, after the addition of dimeric biacetyl, was heated for 15 min. at 90°, clarified by filtration, and adjusted to pH 1 by using 12N-hydrochloric acid. The resulting solution was refluxed for 15 min. and cooled; 9-2′-hydroxyethyl-6,7-dimethylisoalloxazine (0·1 g., 21%) separated as orange needles, m. p. 298—300° (lit.,[19] 300—301°). The infrared and ultraviolet spectra of this material were identical with those of an authentic specimen.

Similar cyclisation was brought about at various pH values in the range 1—6, and at pH 13.

(b) *From trimeric biacetyl*.[9] 2,6-Dihydroxy-4-2′-hydroxyethylamino-5-nitropyrimidine (0·25 g.) was reduced by sodium dithionite as above. 12N-Hydrochloric acid was added to pH 4, followed by trimeric biacetyl [9] (0·25 g.), and the mixture was heated for 10 min. at 90°. Chromatography of the mixture showed the presence of two pteridines identical with those described in (*a*) above, plus a third compound which was shown to be 8-2′-hydroxyethyl-6,7-dimethyl-lumazine by comparison with an authentic sample.

Direct cyclisation of the intermediate pteridine (VII; R = CH_2·CH_2·OH) was effected as above by refluxing a solution of it for 15 min. at pH 1. On cooling, the isoalloxazine separated as orange needles (23%), m. p. 299—300°. The mother-liquors contained 8-2′-hydroxyethyl-6,7-dimethyl-lumazine which was separated by large-scale paper chromatography, plus small quantities of the pteridine (IX; R = CH_2·CH_2·OH).

Lumiflavin (I; R = Me).—(a) *From dimeric biacetyl* (VI). 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0·23 g.) was reduced and condensed with dimeric biacetyl (0·5 g.) as above. The intermediate pteridine (VII; R = Me) readily cyclised when the solution at pH 1 was refluxed for 30 min. Lumiflavin (I; R = Me) was obtained by chloroform-extraction of the resulting yellow solution, followed by evaporation of the extract, to give orange needles (0·03 g.) identical with authentic lumiflavin.[20] We believe that this cyclisation procedure is superior to the method of Birch and Moye [10] who used polyphosphoric acid.

The aqueous layer from the above chloroform-extraction deposited an orange solid (0·035 g.)

[19] Fall and Petering, *J. Amer. Chem. Soc.*, 1956, **78**, 377.
[20] Kuhn, Rudy, and Wagner-Jauregg, *Ber.*, 1933, **66**, 1950.

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overnight. Recrystallisation from water gave 6,8-*dimethyl*-7-(2-*methyl*-3-*oxobut*-1-*enyl*)-*lumazine* (IX; R = Me) as orange needles, m. p. 258—262° (Found: C, 55·5; H, 5·1; N, 20·2. $C_{13}H_{14}N_4O_3,0{\cdot}5H_2O$ requires C, 55·2; H, 5·3; N, 19·8%). As in the case of the hydroxyethyl analogue, this compound was not converted into the isoalloxazine by acid or alkali.

(b) *From trimeric biacetyl.*[9] Similar reduction of the nitropyrimidine (0·2 g.) followed by condensation with trimeric biacetyl (0·28 g.) and cyclisation at pH 1 for 1 hr. gave lumiflavin as bright orange needles (0·04 g., 30%), m. p. 326° (lit.,[20] 328°). The mother-liquors were shown by paper chromatography to contain 6,7,8-trimethyl-lumazine plus small quantities of the pteridine (IX; R = Me).

Riboflavin (I; R = D-ribityl).—(a) *From dimeric biacetyl* (VI). 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine (0·2 g.) was reduced with sodium dithionite, and subsequently condensed with dimeric biacetyl (0·25 g.) as above. Paper chromatography of the resulting solution showed the presence of two pteridines, which from their spectral characteristics (see Table) are assigned structures (VII; R = D-ribityl) and (IX; R = D-ribityl). The former was readily cyclised by heating the mixture for 30 min. at pH 1 and, on cooling, riboflavin (0·016 g.) separated as orange needles, m. p. 289° (lit.,[16] 292°). The infrared and ultraviolet spectra of this material were identical with those of authentic riboflavin.

Similar cyclisation was brought about at various pH values in the range 1—6 and at pH 13.

(b) *From trimeric biacetyl.*[9] Similar reduction of the nitropyrimidine (0·25 g.) followed by condensation with biacetyl trimer (0·25 g.) and cyclisation at pH 1 gave riboflavin (0·03 g., 20%) as orange needles, m. p. 289°. The mother-liquors were shown to contain 6,7-dimethyl-8-D-ribityl-lumazine (VIII; R = D-ribityl) plus small quantities of the pteridine (IX; R = D-ribityl).

Lumichrome.—To a solution of trimeric biacetyl[9] (1·85 g.) in water (40 c.c.) at about 80° was added 4,5-diamino-2,6-dihydroxypyrimidine sulphate[21] (2·45 g.). The mixture was heated for a few minutes, treated with charcoal, filtered rapidly, and kept overnight at 0°. The crude product was then collected and recrystallised twice from water, to give 7-(2-hydroxy-2-methyl-3-oxobutyl)-6-methyl-lumazine as light yellow prisms (0·6 g.), m. p. 230—232° (lit.,[7] 230—232°) (Found: C, 51·5; H, 5·2. Calc. for $C_{12}H_{14}N_4O_4$: C, 51·8; H, 5·1%).

The mother-liquors were shown to contain 6,7-dimethyl-lumazine by paper chromatography and comparison of the ultraviolet spectrum with that of an authentic sample.[7]

Lumichrome was obtained by heating 7-(2-hydroxy-2-methyl-3-oxobutyl)-6-methyl-lumazine (0·35 g.) in 0·1N-sodium hydroxide (35 c.c.) for 1 hr. at 100°. Acidification of the mixture with 2N-hydrochloric acid gave lumichrome (0·29 g.) as a bright yellow solid, m. p. >300°. The ultraviolet and infrared spectra were identical with those of an authentic sample.[22]

This pteridine could not be cyclised by using acid in the pH range 1—6.

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[21] Bogert and Davidson, *J. Amer. Chem. Soc.*, 1933, **55**, 1667.
[22] Karrer, Salomon, Schöpp, Schlittler, and Fritzsche, *Helv. Chim. Acta*, 1934, **17**, 1010.

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