CHEMICAL STUDIES OF THE BIOSYNTHESIS OF RIBOFLAVIN

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A T H E S I S

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A C K N O W L E D G M E N T S

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OBJECTIVES

The elusive nature of riboflavin 2-imine and the intriguing possibility that it was a biological precursor of riboflavin made the synthesis of this new type of isoalloxazine an attractive problem. It was therefore decided that a new attempt at the synthesis of riboflavin 2-imine and related problems should be studied.

Having synthesised riboflavin 2-imine and having established that it was not a precursor in flavinogenesis, it became obvious that the outstanding intermediate in riboflavin biosynthesis was 5-amino-2,6-dihydroxy-4-D-ribitylaminopyrimidine. The synthesis of this vital pyrimidine was undertaken with a view to investigating its chemical transformation to riboflavin under conditions of possible biological significance.

INDEX

HISTORICAL

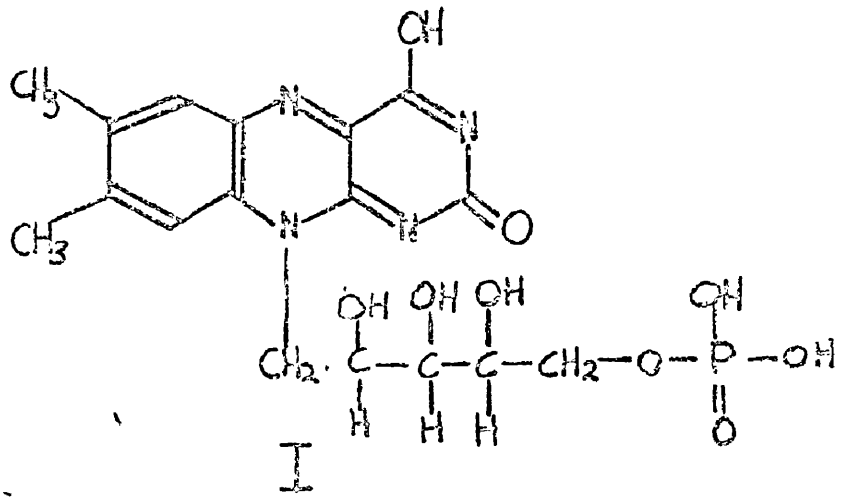
INTRODUCTION

Riboflavin is one of the most widely distributed vitamins and appears to be present in small amounts in each animal and plant.[1] Its synthesis by organisms appears to be common and five synthesise it in large amounts.

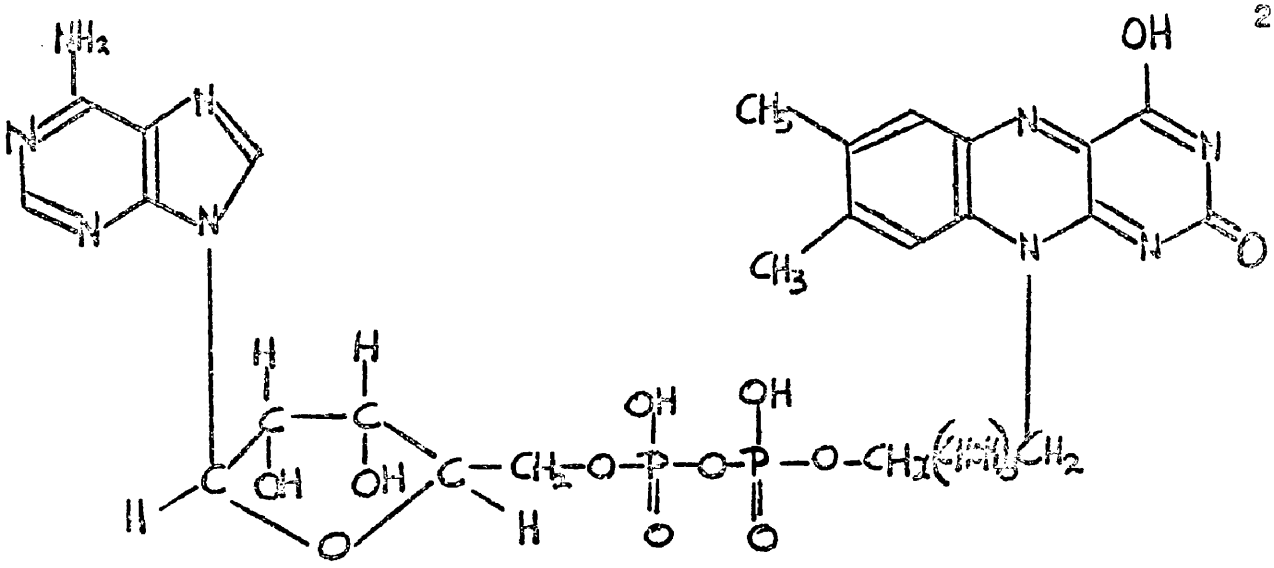
1. Clostridia acetobutylicum[2]
2. Mycobacteria smegmatis[3]
3. Candida species[4]
4. Eremothecium ashbyii[5]
5. Ashbya gossypii[6]

The last two in particular have been used for the biosynthetic studies which are discussed in a later section.

The main role of riboflavin in organisms is as the prosthetic nucleotide portion of the flavo-protein enzymes, of which between 20 and 30 are at present known. A number of these have riboflavin-5'-phosphate (I, flavin mononucleotide or FMN) as the prosthetic group, but the majority contain flavin-adenine dinucleotide (II, FAD).



I



II

The riboflavin portion of these structures is readily reduced by chemical reagents and in so doing adds two hydrogen atoms across its quinonoid structure to give the leuco compound.[7] These leuco compounds are readily reoxidised by oxygen.[7]

The biological activity of the flavin nucleotides when incorporated in the flavo-proteins is due to this oxidation and reduction, in other words, they act as hydrogen carriers. The riboflavin portion of a flavo-protein enzyme is readily reduced by its specific substrate, e.g., one of the reduced co-enzymes. In some cases the flavin group is not re-oxidised by O_2, but by specific acceptors such as cytochrome c. Such cases are the Coenzyme I- cytochrome c and Coenzyme II-cytochrome c reductases, the first of which occurs in bacteria[8] and the other in animal tissues and yeast.[9,10,11] In other cases the flavin group reacts directly with oxygen which is

reduced to hydrogen peroxide, such enzymes are oxidases like the D-aminoacidoxidases, which occur in liver, kidney, moulds, and bacteria, and in which the flavin is the prosthetic group in association with iron.[12] The reaction in this case is

$$\text{D-amino acid} + O_2 \longrightarrow \alpha\text{-keto acids} + NH_3 + H_2O_2$$

Albert[13,14] pointed out that riboflavin contained a grouping resembling the well-known metal chelating compound 8-hydroxy quinoline, and showed that it has the property of binding divalent ions of heavy metals, in particular Fe^{++}. These chelates contain two metal atoms per molecule of riboflavin,[15] but in flavo-protein enzymes the ratio is 1:1.[15] All such metal containing enzyme systems are dehydrogenases. It has been shown that while the metal is necessary for the reduction of some of the acceptors, it is not necessary for others. Thus it is often possible to remove the metal by dialysis against, say, 8-hydroxy quinoline so giving a metal-free enzyme which has lost its ability to react with cytochrome c, ferricyanide or nitrate, but which still retains its original ability to reduce O_2, dyes, quinones or co-enzymes. The metal is therefore shown to be not concerned with the activation of the hydrogen donor.[16]

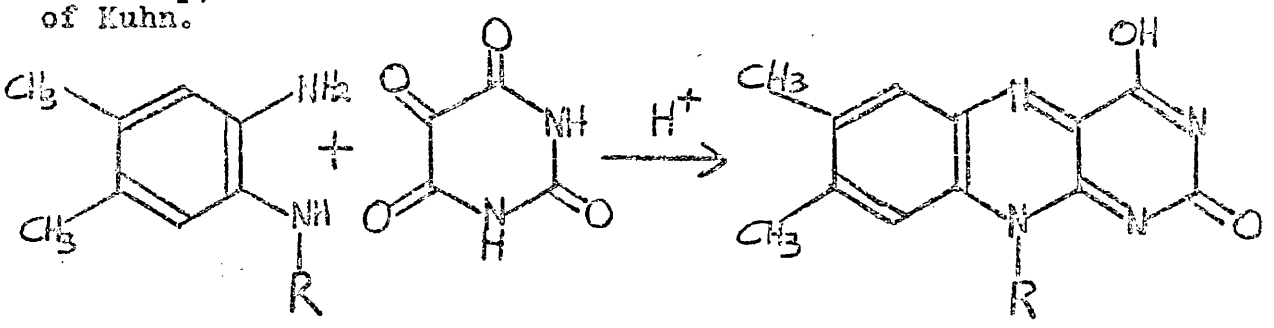
Synthesis of isoAlloxazines.

The important step in all isoalloxazine syntheses consists of the condensation of an aromatic component (Ring A) with a pyrimidine component (Ring C) thus forming the pyrazine ring (Ring B) giving the typical fused three-ring system.

The problem of total synthesis consists therefore in defining the proper condensation conditions as well as the preparation of the required aromatic and pyrimidine intermediates.

isoAlloxazine syntheses can best be classified into three distinct types.

1. Condensation of an o-phenylene-diamine type compound (III) with alloxan (IV) in acid conditions after the manner of Kuhn.[17]



III IV V

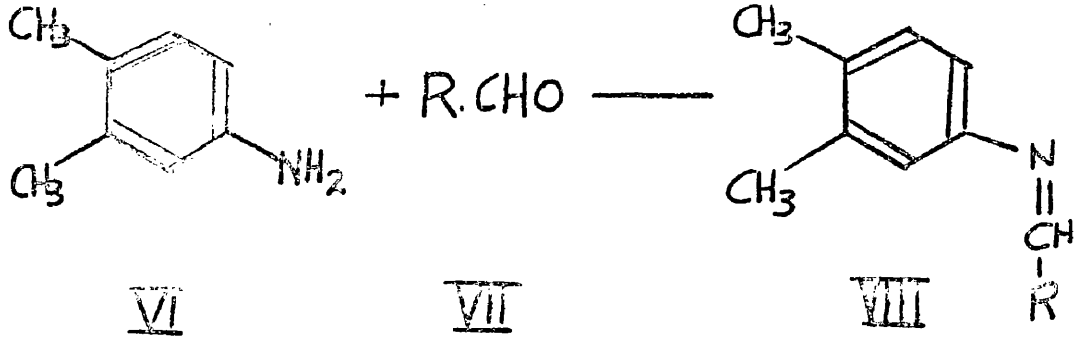
This was the method by which the first syntheses of lumiflavin (V, R = Me) and riboflavin (V, R = D-Ribityl) were achieved by Kuhn, Weygand,[18] Karrer[19] and their many co-workers. The weakness of the method resides in the oxygen-sensitivity of the required o-phenylene-diamines, which

is so pronounced that these vital intermediates are best never isolated. The condensation of these o-phenylene-diamines with alloxan in the presence of boric acid takes place at room temperature in most cases in good yield.

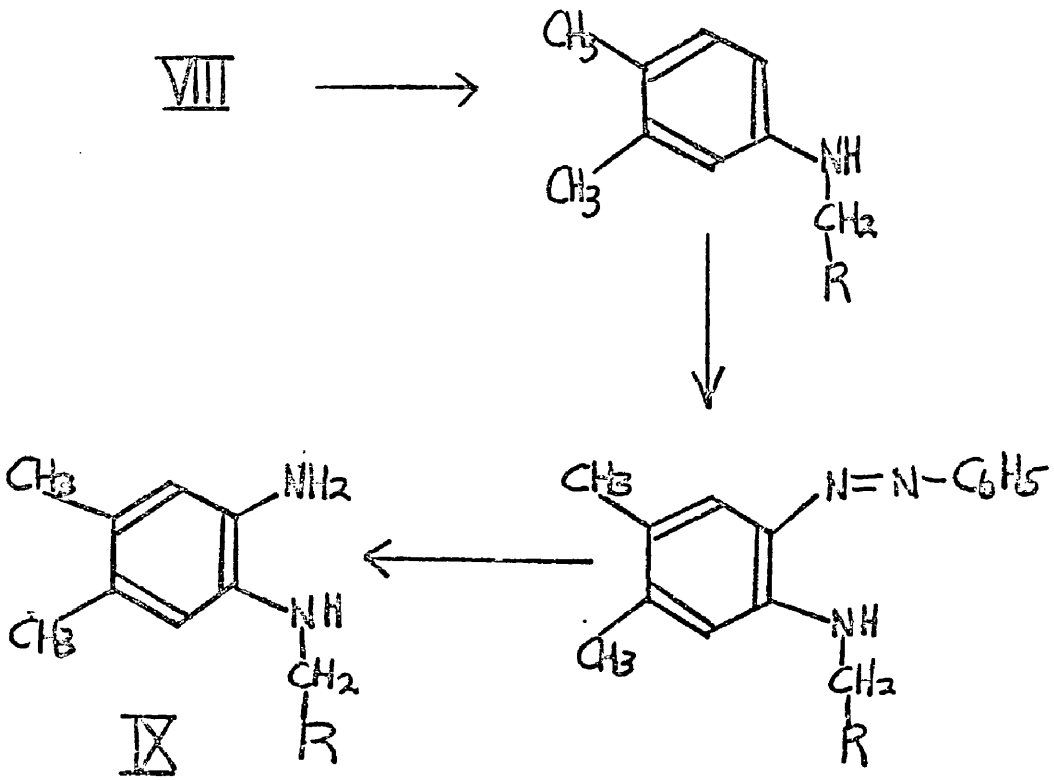
It is obvious at this stage that such variations as there are in the above syntheses of isoalloxazines lie in the preparation of the required o-phenylene-diamine. In the case of the synthesis of riboflavin the required compound is 2-amino-4,5-dimethyl-1-D-ribitylaminobenzene (III, R = D-Ribityl), and three of the most profitable and interesting routes to this compound are summarised below.

(a) 3,4-Xylidine (VI) is condensed with D-ribose (VII, R = $-(CHOH)_3.CH_2OH$) and the resulting riboside (VIII) is reduced catalytically.[19] The introduction of the second amino group is achieved by coupling with diazonium salts to form the azo dyes, which on reduction, either catalytically or chemically, give the required diamine (IX, R = $-(CHOH)_3.CH_2OH$).[19,20]

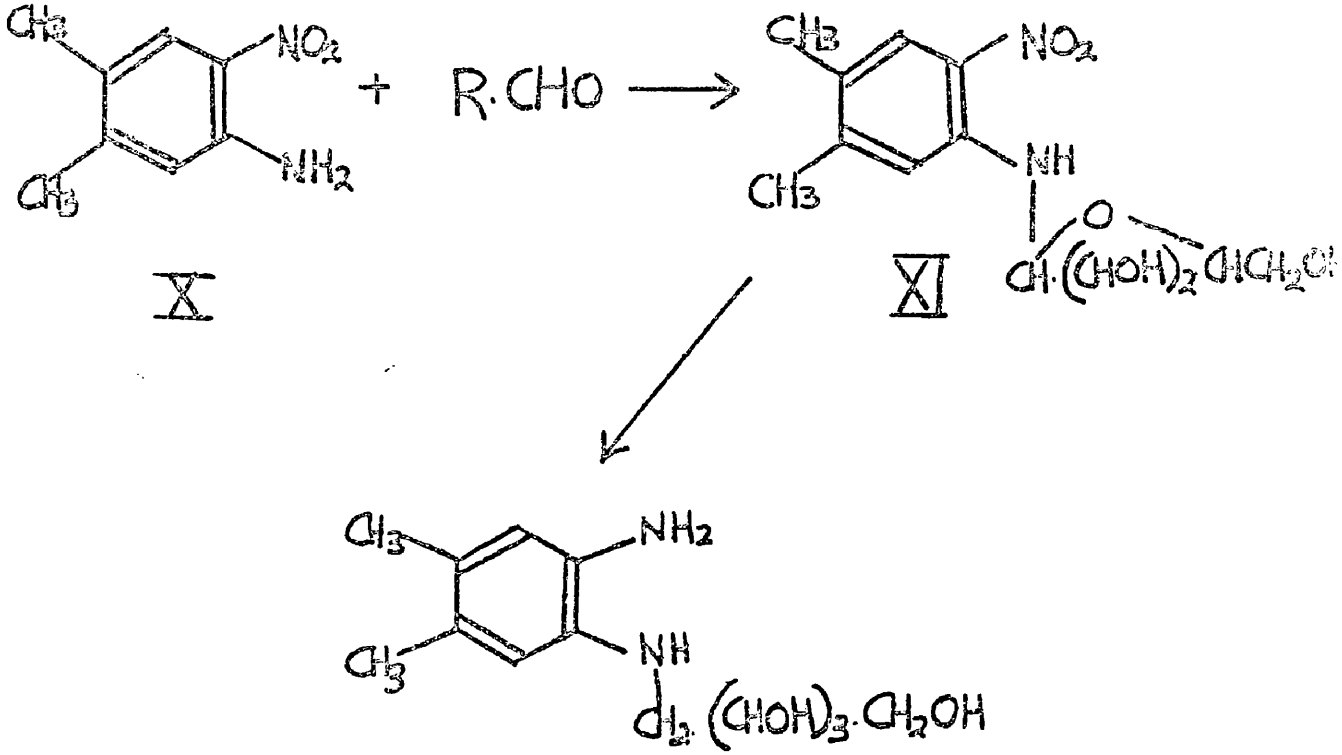
This method gives a 38% yield of riboflavin based on the initial D-ribose.



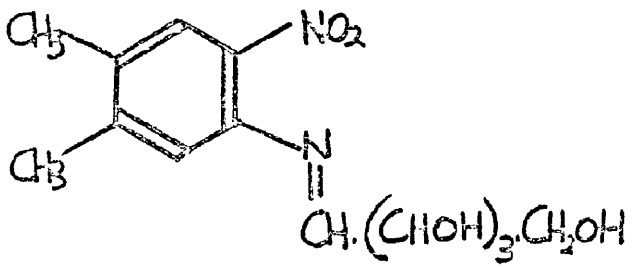
VI VII VIII



(b) o-Nitro-3,4-xylidine (X) is condensed with D-ribose and the reaction product (XI) is reduced catalytically to the diamine.[21,22] Using this method yields of 16% of riboflavin based on D-ribose are obtained.

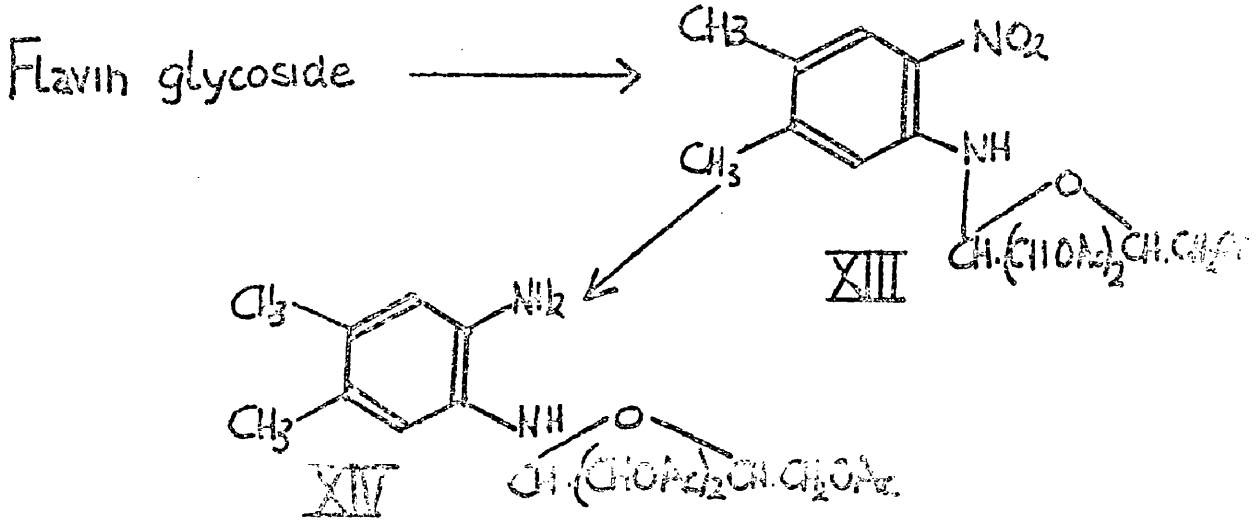


The intermediate amino-glycoside (XI) exists in equilibrium with its tautomeric Schiff base (XII).



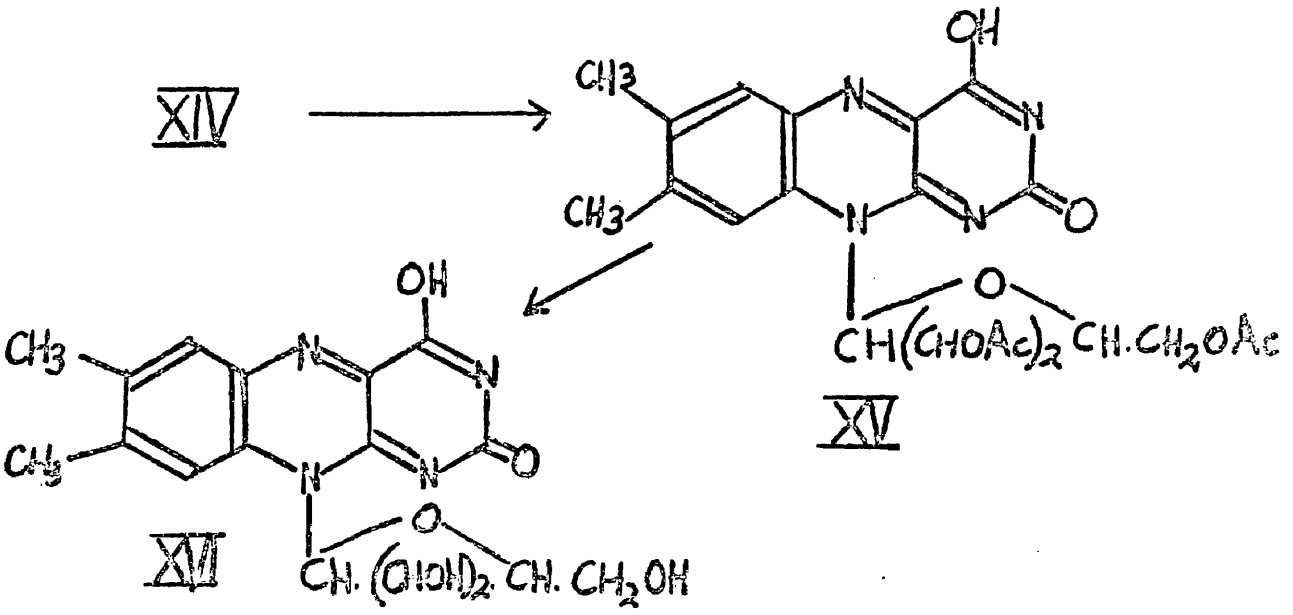
XII

The amino-glycoside affords a synthesis of the isoalloxazine-glycosides as it may be reduced exclusively at the nitro group. The synthesis is achieved by first acetylating the amino-glycoside to give its tri-acetate (XIII) which is partially reduced to the diamine (XIV) which in turn is condensed with alloxan to give the tri-acetate (XV) of the isoalloxazine-glycoside.[23] The free glycoside (XVI) is obtained from this by saponification, and it is of interest that the isoalloxazine-glycosides do not show the physiological properties of vitamin B_2.



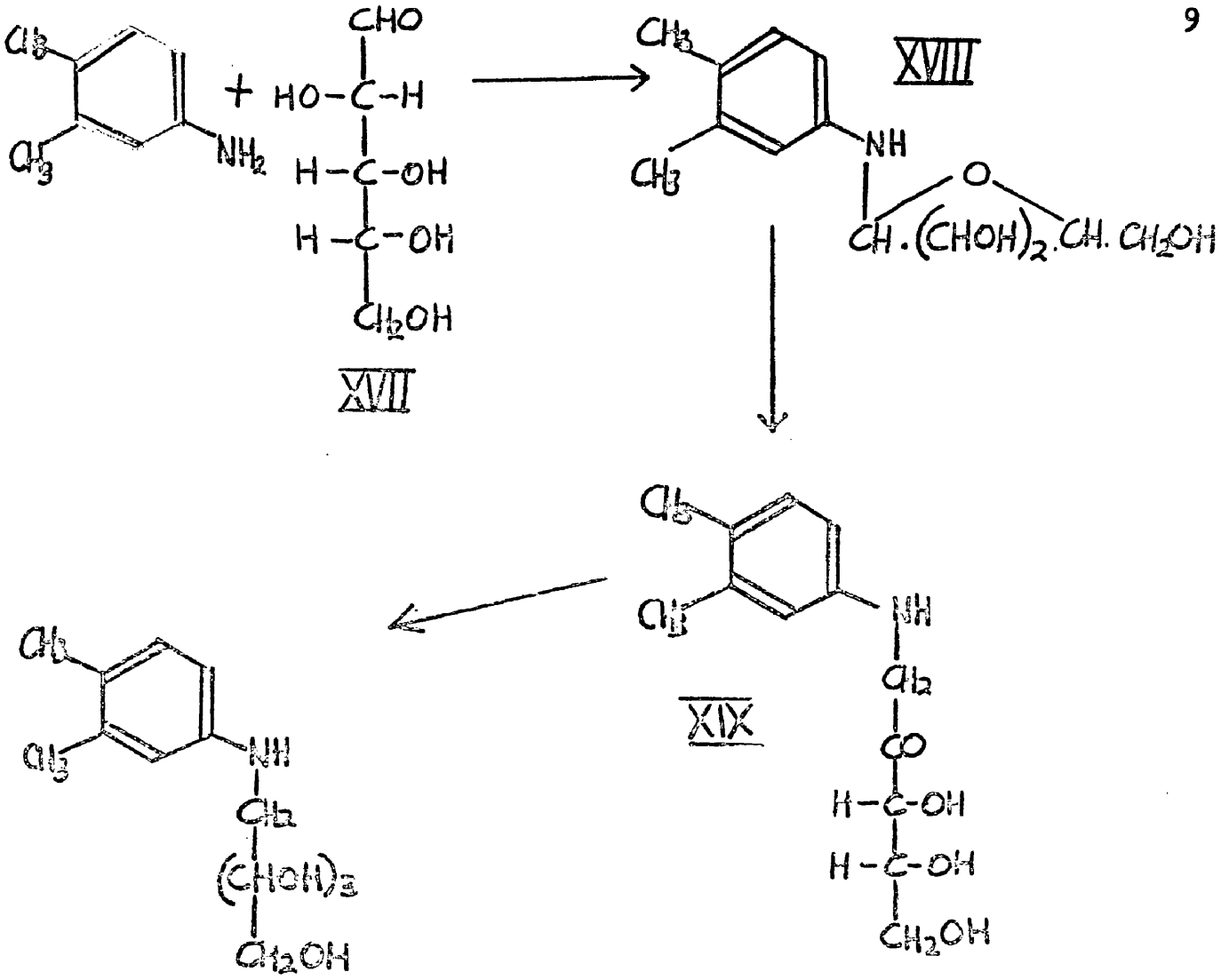
XIII

XIV



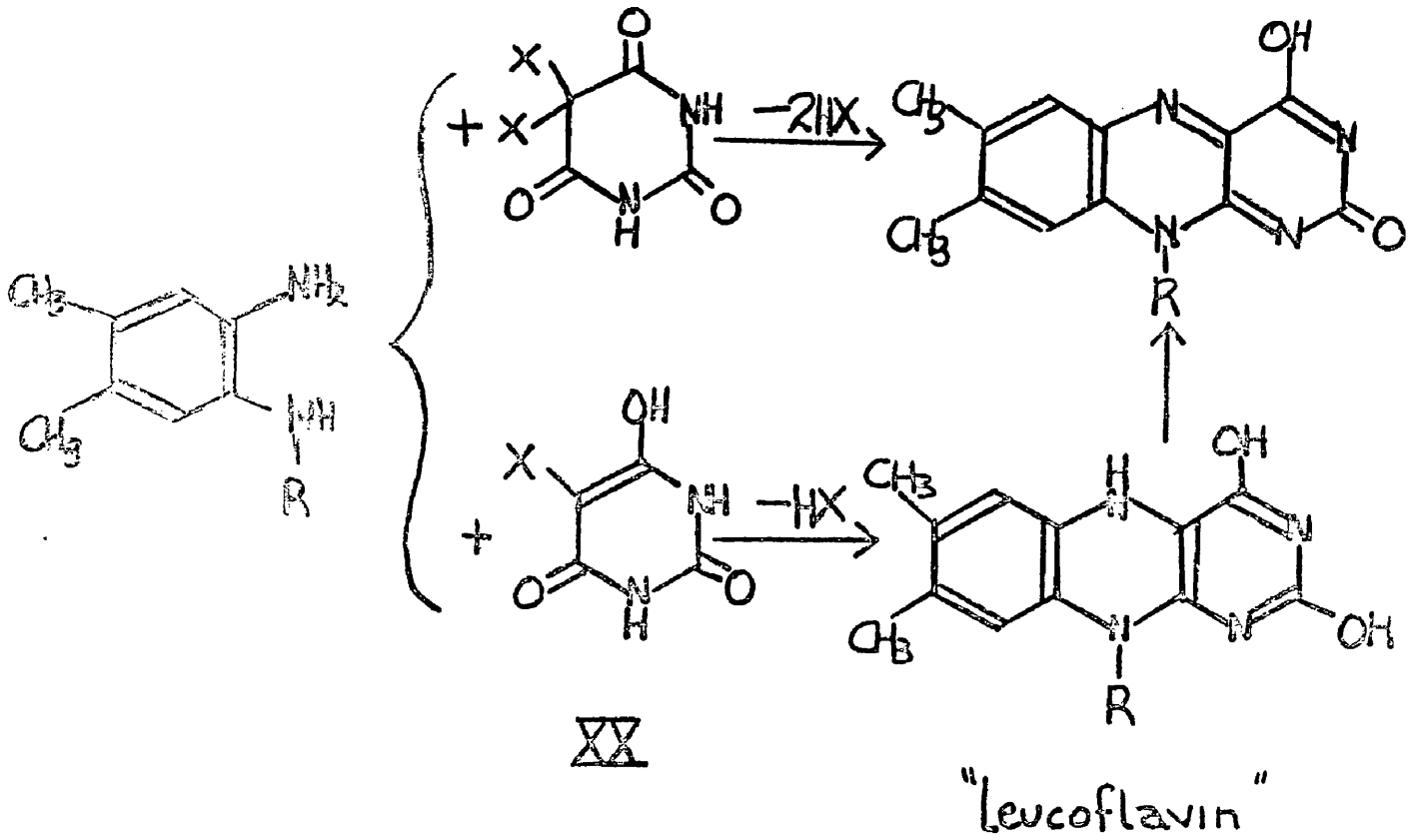
(c) Procedures (a) and (b) both use D-ribose as the sugar source in the synthesis; it is also possible to use the less expensive D-arabinose (XVII) and this is the basis of perhaps the most elegant of isoalloxazine syntheses.[24] In this procedure D-arabinose is condensed with 3,4-xylidine in the presence of small amounts of acid to give the D-arabinoside (XVIII) which undergoes an Amadori-rearrangement upon heating to 75° to form the D-iso-arabinosamine (XIX). Catalytic hydrogenation in alkaline medium converts the D-iso-arabinosamine to 4,5-dimethyl-1-D-ribitylaminobenzene. The yield of this intermediate is 13% based upon the initial D-arabinose. The procedure from then is as above in (a).

It is worth noting that the reduction of the keto group in the sugar side chain of the Amadori rearranged product gives stereo-specifically the desired isomer.



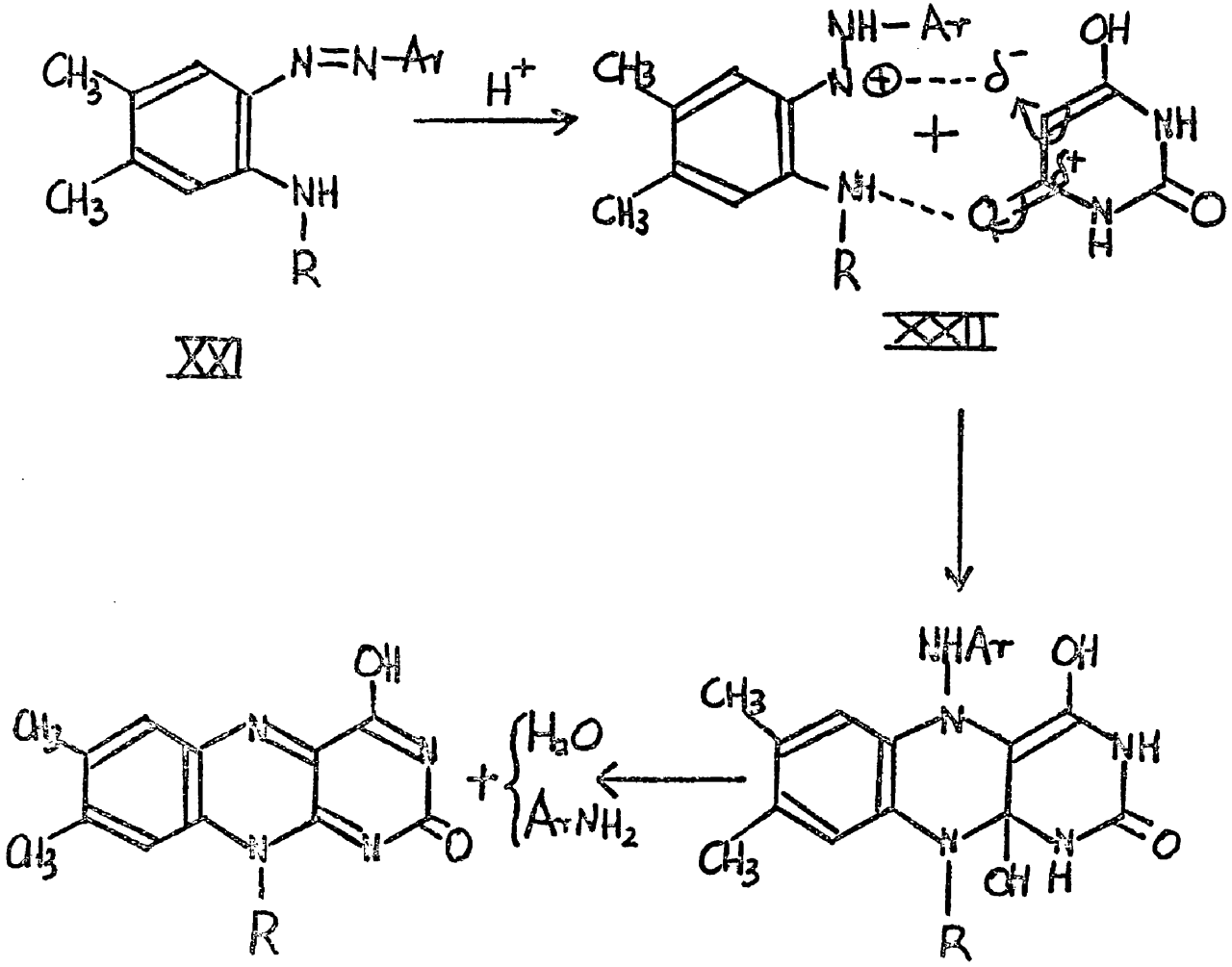
2. The second general method of isoalloxazine synthesis is commonly known as the Ladenburg synthesis. This route consists of condensation of the above 2-amino-4,5-dimethyl--1-D-ribitylaminobenzene with 5-substituted mono- or di- halo barbituric acids (XX, X = halogen) in pyridine.[28] This procedure is less mild and more cumbersome than the first general method.

Halogen substituted barbituric acids are however more readily accessible than alloxan. The yields in the reaction are about the same as in (1) and the reaction



proceeds using either a mono- or a di- substituted halogen compound or a mixture of the two.

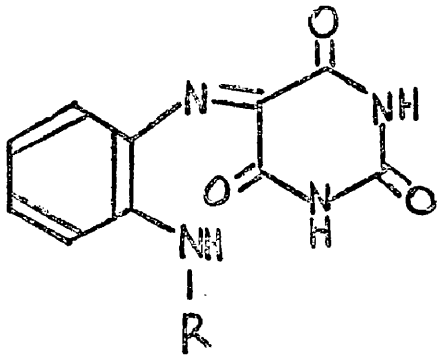
3. The third method was likewise discovered by Tishler and is called the "Tishler Condensation". This procedure consists of condensation of an o-secondary amino-azo compound (XXI) with free barbituric acid.[26] The reaction takes place in excellent yield in a refluxing butanol acetic acid mixture. Although the reaction, which involves the breaking of N=N double bond, under these conditions is unusual Hemmerich[27] believes it is possible to show it as electrophilic substitution after the following scheme.



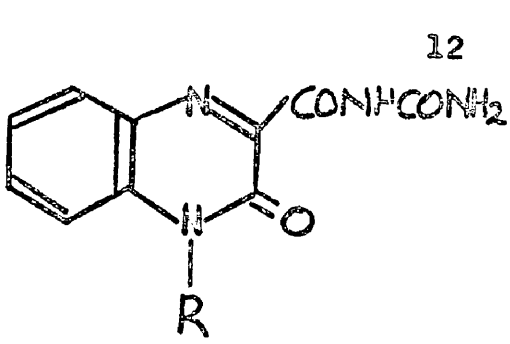
XXI

XXII

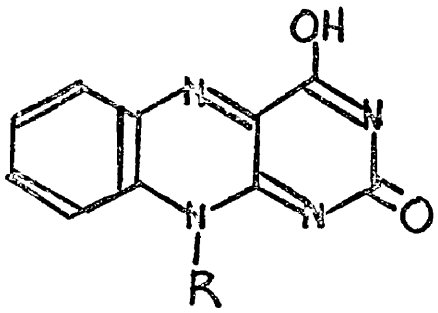
Hemmerich found that with higher acidity there is acceleration in the reaction rate and from this he implies that the protonised azo-body (XXII) must be the reactive intermediate. That the above mechanism is a realistic one is supported by the fact that Kuhn and Weygand[18] found that the product of the condensation of an o-phenylene-diamine with alloxan in neutral solution could not subsequently be cyclised to an isoalloxazine (XXV). Kuhn and Weygand believed that this product was the anil (XXIII), but more recently King and Clark-Lewis[28] have discarded this structure and have alternatively proposed that of a quinoxaline-carboxyureide (XXIV).



XXIII



XXIV

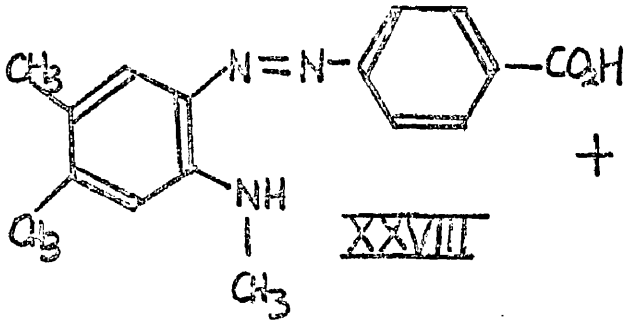


XXV

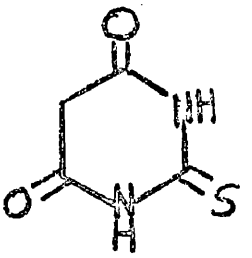
Recently Hemmerich et al.[29,30,31] have been responsible for several interesting syntheses of isoalloxazines, each of which has been an extension of a known method. These are summarised below.

(a) The condensation of thiobarbituric acid (XXVI) and iminobarbituric acid (XXVII) with N,3,4-trimethyl-6-(p-carboxyphenyl azo)-aniline (XXVIII) gives 2-thiolumiflavin (XXIX) and 2-lumiflavimine (XXX) respectively.[29]

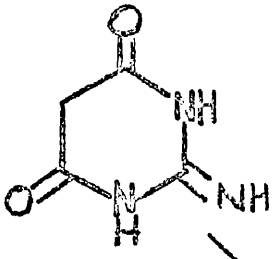
A more elegant synthesis of 2-lumiflavimine is achieved by the reduction, using Raney nickel, of N,4,5-trimethyl-2-nitraniline (XXXI) followed by condensation of the



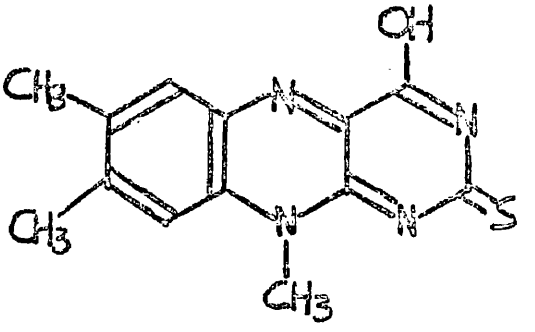
XXVIII



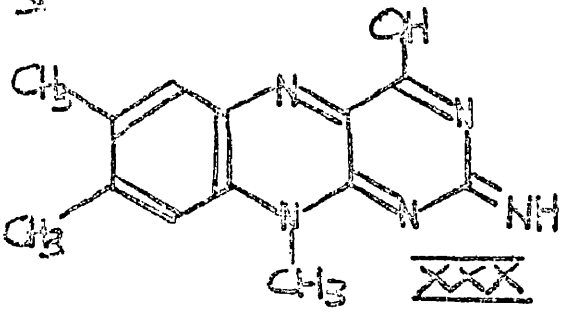
XXVI



XXVII

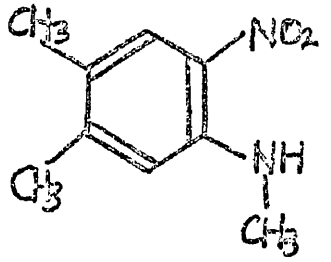


XXIX

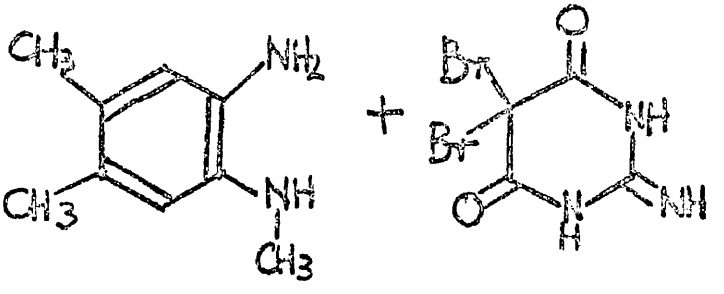


XXX

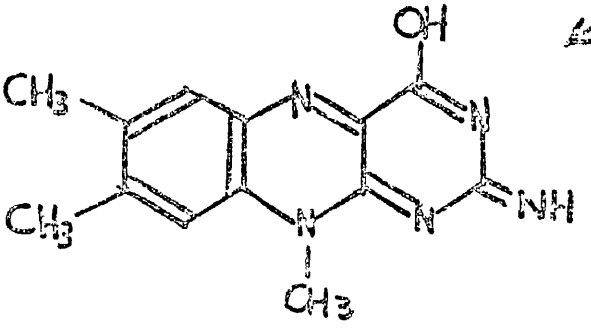
resulting o-phenylene-diamine with 5,5-dibromobarbituric acid-2-imine (XXXII).[30]



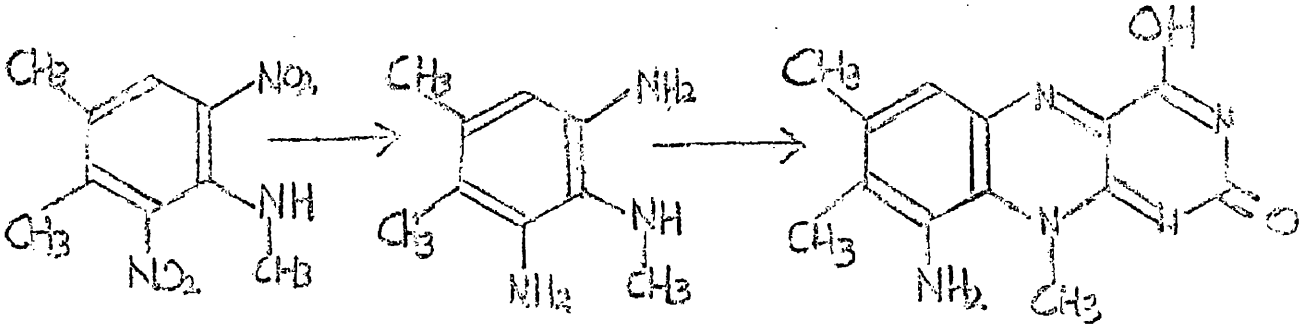
XXXI



XXXII

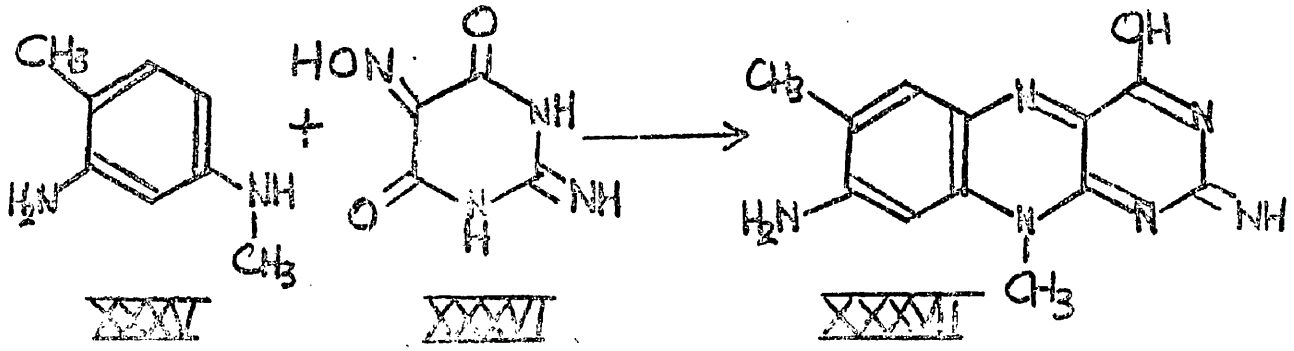


(b) Another intersting variation is achieved by inserting new groups in the benzenoid intermediate and then condensing with alloxan. By this method 8-aminolumiflavin (XXXIII) is produced from N,3,4-trimethyl-2,6-dinitraniline (XXXIV).[30]



XXXIV XXXIII

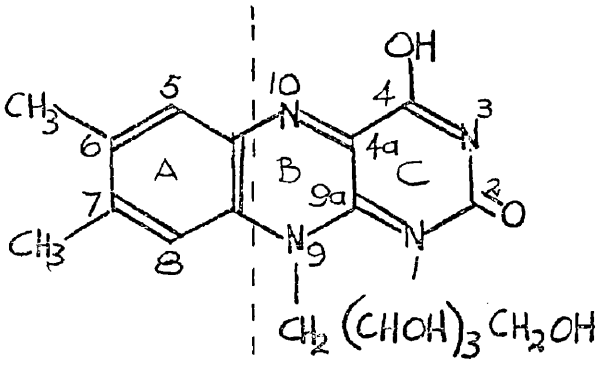
(c) The latest variation used by Hemmerich is based on the condensation of substitued 2,4-diaminotoluenes with derivatives of violuric acid.[31] The resulting 7-amino iso-alloxazines are interesting compounds in as much as they are appreciably more stable than the normal 7-methyl derivatives. Typical of this type of reaction is the condensation of 2-amino-4-methyl amino toluene(XXXV), formed from its 2-nitro analogue by catalytic reduction, with violuric acid-2-imine (XXXVI) to give 8-nor-8-amino-2-lumiflavimine (XXXVII).[31]



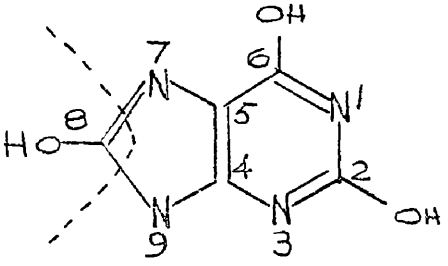
XXXV XXXVI XXXVII

The Biosynthesis of Riboflavin.

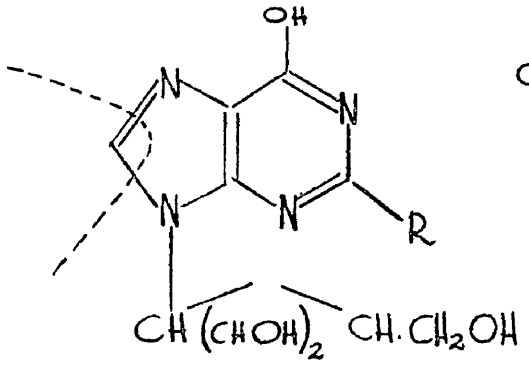
Comparison of the structural formula of riboflavin with those of the naturally occurring purines and pteridines shows a striking resemblance in that they all have as a part of their nucleus the skeleton of a diaminopyrimidine. In the examples below this is shown clearly on the right hand side of the dotted line. On the basis of this structural similarity it would not be surprising if there were some close biological relationship between these three important groups of compounds.



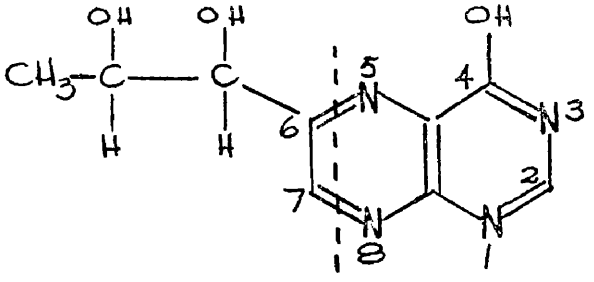
Riboflavin



Uric acid



R = NH_2, Guanosine
R = OH, Xanthosine



Biopterin

MacLaren[32] was the first to suggest that purines played a role as precursors in the biosynthesis of riboflavin. This hypothesis has been confirmed by many workers, and from a combination of their efforts a picture has taken shape which is at once both exciting and intriguing.

Results obtained by Plaut[33] on the incorporationg of radioactively labelled compounds into riboflavin using Ashbya gossypii showed a striking similarity to results obtained by Buchanan, Sonne and Delluva[34,35] on the incorporation of similarly labelled compounds into uric acid. Thus, ^{14}C-formate enters position 2 of riboflavin and positions 2 and 8 of uric acid, ^{14}C-carbon dioxide or $CH_3{}^{14}CO_2H$ enters position 4 of riboflavin and position 6 of uric acid, and the carbon atoms of glycine enter positions 9a and 4a of riboflavin and positions 4 and 5 of uric acid. The nitrogen of glycine enters position 7 of uric acid, and although no degradation has as yet been worked out that will give the contribution to the individual nitrogen atoms in riboflavin, nitrogens 9 and 10 contain thirty times the amount of label carried by nitrogens 1 and 3. Support for these incorporation studies came from the work of Goodwin and his group who showed that serine stimulated flavinogenesis[36,37] and that before its incorporation it was transformed to formate and glycine,[37] and from Klungsoyr[38] who demonstrated the

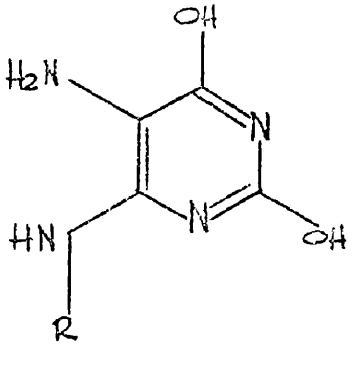
incorporation of labelled formate into the 2 position of riboflavin. Goodwin also showed[37] that the incorporation of labelled serine could be diluted out using adenine.

The most striking evidence for the conversion of purines into riboflavin came from the work of McNutt[39,40] who, working with adenine and the mould Eremothecium ashbyii, showed that in the transformation carbon atom 8 of the purine was "lost". This result he confirmed both with adenine specifically labelled in the 8 position,[39] and with adenine which was randomly labelled.[40] From his results he came to the conclusion that the contribution of the purine to riboflavin was an intact pyrimidine ring, and this appears to be generally accepted. The present author, however, was struck by the fact, first observed by McNutt[39] but later ignored, that there seemed to be some evidence for opening and possible "loss" in the pyrimidine ring of the purine. This evidence is discussed in the appendix to this section.

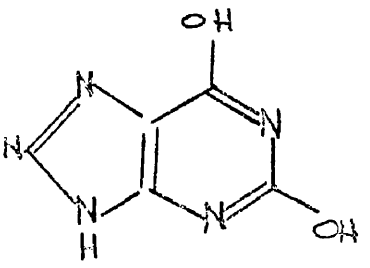
Leaving aside the question of whether or not the pyrimidine ring opens the fact that the essential contribution of purines to riboflavin is an intact pyrimidine ring is supported by Goodwin's suggestion[41] that 4,5-diaminouracil is a normal metabolite of E. ashbyii. Diaminouracil is not however an intermediate in riboflavin biosynthesis as Goodwin,[42] Korte,[43,44] and Masuda[45] have all searched in vain for a

stimulation of flavinogenesis, and indeed Goodwin has been able to show[42] that this compound, which is normally very sensitive to oxidative self-condensation, undergoes such a reaction in the culture medium. It is generally accepted that the vital intermediate in the transformation of purines is in fact the ribitylaminopyrimidine (XXXVIII, R = D-ribityl) even although this compound has never been isolated or detected *in vivo*. This failure to detect such an important precursor is, however, not surprising as it undergoes self-condensation very rapidly. This is discussed further in the theoretical section of part two of this thesis.

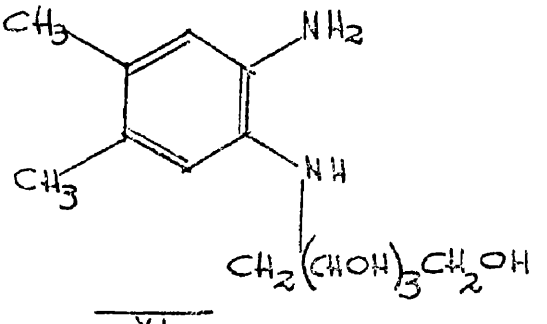
Two other points concerning the purine contribution to flavinogenesis must be mentioned. The first of these is that the aza-purines all inhibit flavinogenesis,[42,46] which is to be expected, and that the one corresponding to xanthine (XXXIX) does so preferentially.[42] The second point is that the only reasonable alternative route to riboflavin, that from 2-amino-4,5-dimethyl-1-D-ribitylaminobenzene (XL), is ruled out owing to the non-stimulatory effect of this compound.[36]



XXXVIII



XXXIX



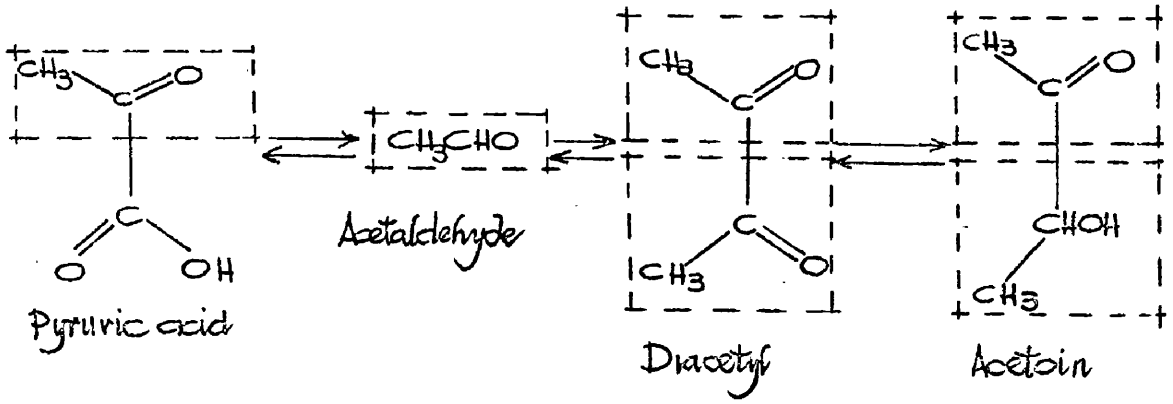
XL

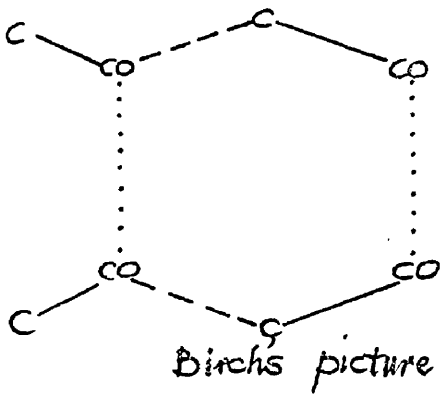
Finally, Goodwin[46] has established that the purine precursors of riboflavin fall into the following order of decreasing effectiveness; guanine, xanthine, adenine, hypoxanthine, and uric acid. He has also shown contrary to McNutt[39] that there is no significant difference between the effectiveness of the parent purine and its riboside. This greater effectiveness of guanine compared with xanthine, coupled with the fact that most of the naturally occurring pteridines have a 2-amino group, raised the possibility that the immediate precursor of riboflavin was its 2-imino analogue. This idea, coupled with the fact that riboflavin 2-imine was reported[47] to be a most elusive compound, stimulated a new attempt at its synthesis and this is the subject of the first part of this thesis. Goodwin, however, reports[48] that riboflavin 2-imine neither promotes nor inhibits flavinogenesis.

The other portion of the riboflavin nucleus which has still to be accounted for is of course the benzene ring, and it is here that the focus of present controversy is situated. Fortunately some extremely elegant work[49,50] with tracer compounds has given a clear picture on which to base subsequent work.

The generally accepted pattern for the formation of the benzene ring is that of Birch[51] who suggests that head to

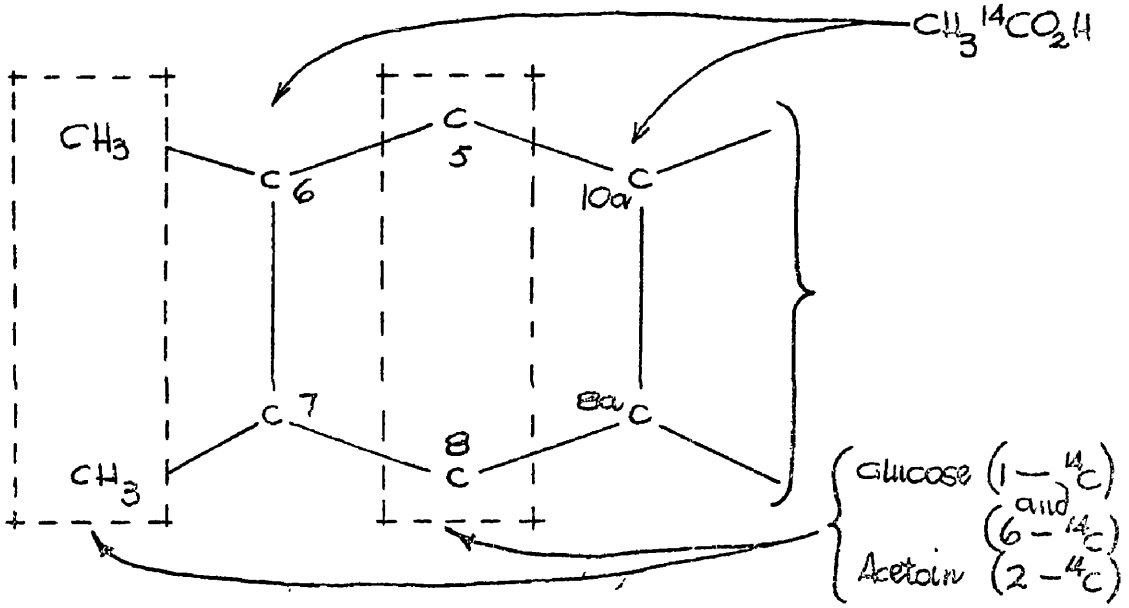
head and head to tail linkages of 4 moles of acetic acid, probably as diacetyl or acetoin derived from 4 moles of pyruvic acid, form the skeleton.





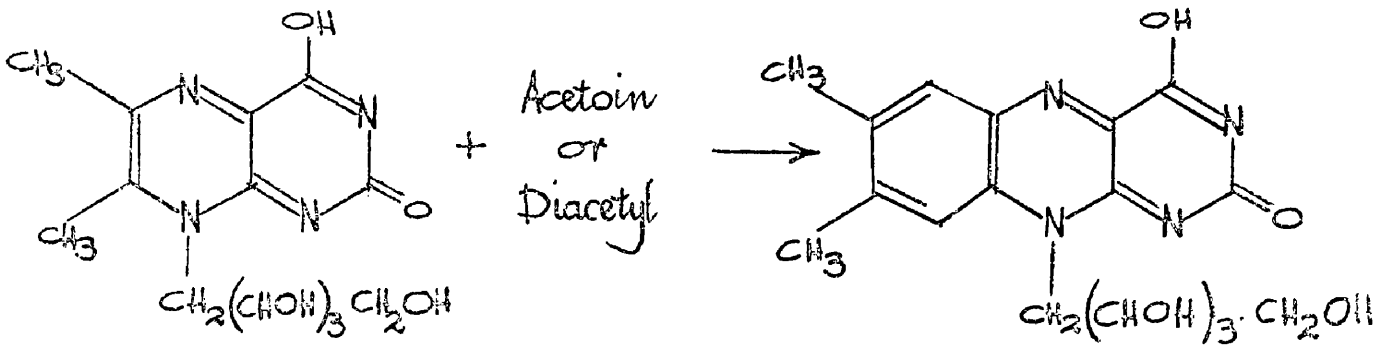
This suggestion is supported by several facts. Masuda[52,53] has been able to identify both acetoin and pyruvic acid in the mycelium and broth of E. ashbyii, and Goodwin[50] has reported the presence of acetoin. Plaut,[49] using incorporation techniques, has been able to demonstrate the following scheme. The label from $CH_3{}^{14}CO_2H$ is incorporated at positions 6 and 7, and positions 8a and 10a of the riboflavin nucleus, and the label from glucose (1-^{14}C) and

glucose (6-^{14}C), which are metabolised to acetate $^{14}CH_3CO_2H$, is incorporated in the methyl groups and in positions 5 and 8. Goodwin also demonstrated[50] that the label from acetoin labelled with ^{14}C in one of its methyl groups is incorporated one half in the methyl groups of riboflavin, and the other half in positions 5 and 8.



The origin of the atoms forming the benzene portion of riboflavin is thus established, but there is considerable uncertainty as to how the benzene ring precursor presents itself to the diaminopyrimidine. Two major hypotheses have been suggested.

Masuda[52,54] was the first to suggest that diacetyl or acetoin reacts with the ribitylaminopyrimidine to give 6,7-dimethyl-ribolumazine (XLI) which he called for convenience G-compound. He then postulates[52] that G-compound reacts with a further mole of diacetyl or acetoin and so gives riboflavin.



XLI

Evidence for this route has gained support from the fact that Masuda[55] and others[43,56] have been able to isolate G-compound from both E.ashbyii and A.gossypii. Plaut has also been able to show[56] using labelled formate and glycine that G-compound has a higher specific radioactivity at early periods of the incubation than riboflavin and is consequently a possible precursor and certainly not a degradation product. The strongest evidence that G-compound is a precursor in the biosynthesis of riboflavin comes from its transformation into riboflavin by various biological systems.[57,58,59,60] On the chemical side Masuda claims that G-compound reacts with diacetyl at 120-130° to give riboflavin,[52] but this type of reaction could not be repeated by Birch and Moye.[61]

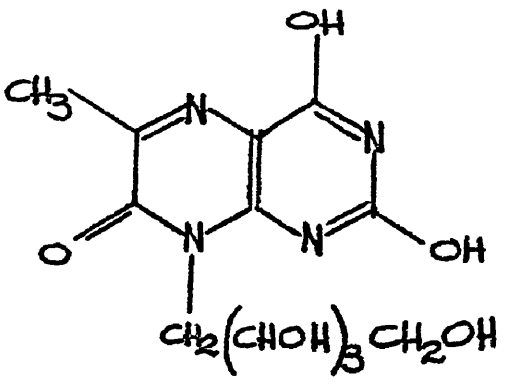
There are, however, almost as many reasons for questioning the validity of this route to flavinogenesis as there are for proposing it. In the first place G-compound is

only present in small amounts compared with riboflavin at any stage in the growth of the mould.[52,56] After 24 hours the ratio of G-compound to riboflavin is 1:50, and after 84 hours it is about 1:30.[52] These ratios indicate an accumulation, however slight, of G-compound, which is unexpected as Masuda[52] has commented on its high activity as an intermediate, and indeed used this argument to explain the small quantity detectable in the culture medium. The quantity of G-compound is indeed so small that McNutt and Forrest[62] failed to observe it during their early work using E.ashbyii although fully aware of Masuda's work.

A critical study of the biological systems used in the transformation of G-compound to riboflavin suggests several points which indicate that an alternative pathway of riboflavin biosynthesis may also operate. Korte[43,44] was the first to report that labelled G-compound isolated from a batch of E.ashbyii which had been inoculated with guanine (5-^{14}C) could not be converted to riboflavin when inoculated into yet another batch of E.ashbyii. Katigiri on the other hand had shown[57] that enzyme solutions or washed cells of E.coli neapolitanus and other organisms converted G-compound to riboflavin in 4½% yield in the presence of pyruvic acid or diacetyl. E.coli neapolitanus, however, is not normally a known riboflavin producing organism and this raises doubts as to what conclusion can be drawn from

this observation. The other biochemical systems which have brought about the conversion of G-compound to riboflavin have been cell-free extracts of A.gossypii[58,59] which gave at the hands of Plaut a 6% and at the hands of Korte a 7% yield, cell-free extracts of E.ashbyii which gave a 7% yield,[59] and cell-free extracts of Mycobacteria smegmatis which gave a yield of 5%.[59] To show how cautiously results using cell-free extracts must be treated Korte has recently pointed out[59] that the biggest conversion of G-compound to riboflavin, i.e. one of 10%, is given by cell-free extracts of M.avium, an organism which has no known riboflavin producing properties. The same worker has also pointed out that guanine which is normally converted to riboflavin by E. ashbyii in around 40% yield is converted not to riboflavin but to xanthine using cell-free extracts of this organism.[59] Other evidence for this tendancy of cell-free extracts not to function like the growing organism seems abundant.[63]

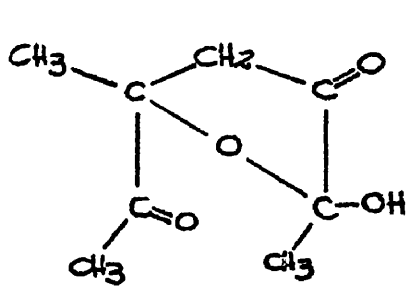
One further interesting compound has been isolated[62,64,65] from riboflavin producing cultures and this has been shown[64,65,66,67] to be 6-methyl-7-hydroxy-ribolumazine (XLII), given the trivial name V-compound by Masuda.



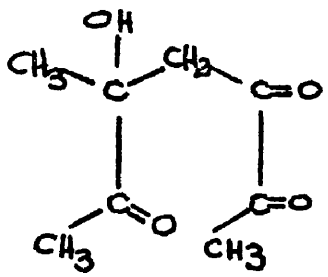
XLII

Masuda,[64] who first isolated this pteridine from E.ashbyii, suggested[66] that it was formed by reaction of pyruvic acid with 5-amino-4-D-ribitylaminouracil, and to support this mechanism reported[63] that the addition of pyruvic acid to E.ashbyii reduced the quantity of G-compound and increased the quantity of V-compound produced. Later work by the same group threw some doubt on this finding for it was shown[68] that V-compound as well as riboflavin was produced when G-compound was incubated with a crude enzyme extract of E.ashbyii. Korte[63] has recently shown that the reason for the low yield mentioned above in the transformation of G-compound to riboflavin using cell-free extracts of various organisms is that G-compound has mainly been converted to V-compound. The role of V-compound, as is that of G-compound, still seems somewhat obscure, but at least in this case there is agreement that it is not an intermediate in flavinogenesis.[62,65,69]

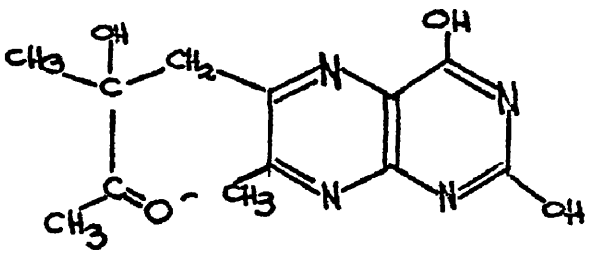
The second hypothesis dealing with the biosynthesis of the benzene ring in riboflavin is based on the chemical studies of Birch and Moye.[51,61] These two workers have shown that two moles of diacetyl can be self-condensed to form an aldol of probable structure (XLIII).[51] This aldol reacts[51] as the hexane trione (XLIV) when heated with either 4,5-diamino-uracil or 5-amino-4-methylaminouracil to give respectively the pteridines (XLV) or (XLVI) or their 7-isomers.[51,61]



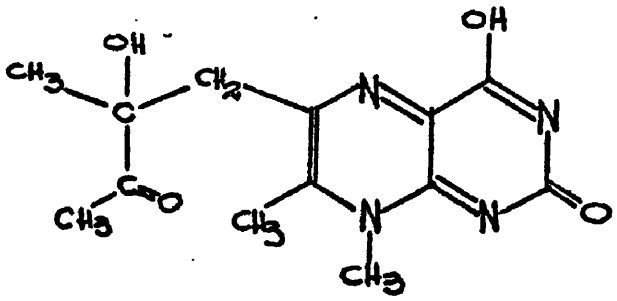
XLIII



XLIV



XLV



XLVI

Further, Birch and Moye demonstrated the cyclisation of the pteridines (XLV) and (XLVI) to give lumiochrome and

lumiflavin respectively.[51,61] The cyclisation of the 8-methyl pteridine was achieved in polyphosphoric acid[61] and the pteridine having no substituent in the eight position was cyclised using N-sodium hydroxide.[51]

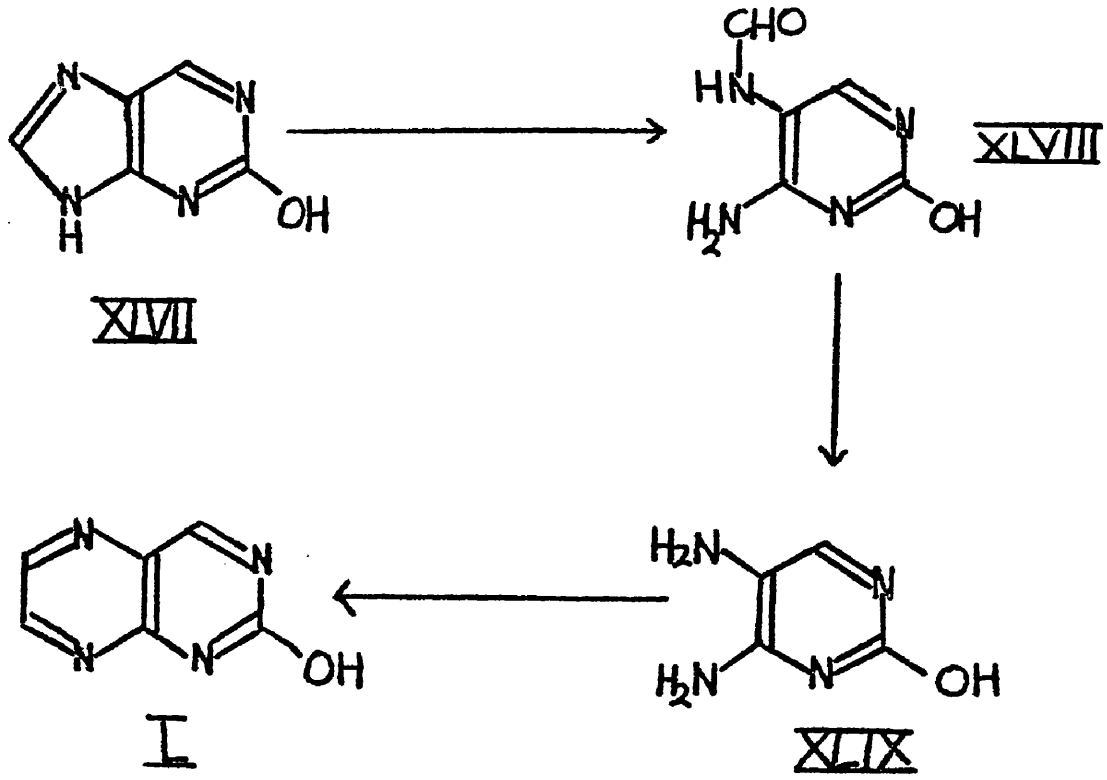
Perhaps the most interesting development in chemical studies of riboflavin biosynthesis has been the one which is the subject of the second part of this thesis, and which may well bridge the gap between the two above-mentioned theories.

The Biosynthesis of Pteridines.

The similarity of the pteridines to riboflavin suggests the possibility of some common pathway in their separate biosynthesis. Support for this hypothesis came from the report by Albert[70] of the in vitro transformation of 2-hydroxy purine (XLVII) to 2-hydroxy pteridine. In this transformation the iminazole ring of the purine opens, at pH5 and 20°, to give 4-amino-5-formamino-2-hydroxy pyrimidine (XLVIII) which then loses its formyl at pH2 to give the 4,5-diaminopyrimidine (XLIX). This 4,5-diaminopyrimidine then condenses with glyoxal, at pH7 and 37°, to give a quantitative yield of 2-hydroxy pteridine (L).

In the light of the transformation of purines into riboflavin, possibly via a pteridine, the order of the following

reaction sequence is of particular interest.

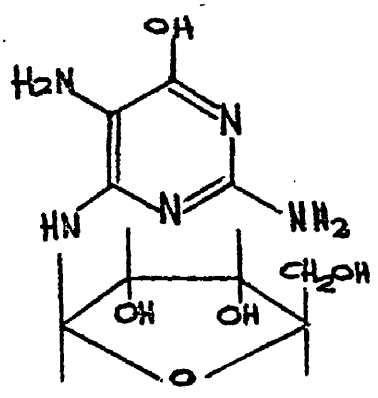


There is also one example of a purine as a pteridine precursor in nature. Ziegler-Gunder et al.[71] injected the larvae of the amphibian Xenopus intradermally with guanine (2-^{14}C). Ten days later the skins of the sacrificed larvae were extracted with N-ammonia at 20°, and chromatography of the extracts yielded several blue-fluorescing spots, one of which was converted by oxidation to labelled 2-amino-4-hydroxy pteridine-6-carboxylic acid.

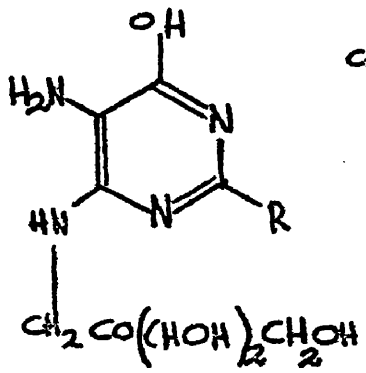
Perhaps the most interesting development in this subject has been the publishing of two independent but very similar theories by Wood[72,73] and Weygand.[74] Wood first

suggested[72] that the ring fission undergone by guanosine and guanylic acid on treatment with alkylating agents, or ionising radiation, may be the initial stage of a purine to pteridine transformation. He predicted that by these means the pyrimidine glycoside (LI) could well be formed.[72] More recently[73] he has advanced the view that this pyrimidine glycoside then undergoes an Amadori rearrangement to give (LII, R = NH_2), which on intramolecular ring-closure would give a pteridine (LIII) similar to biopterin. Alternatively addition of a molecule of water to the 5,6-double bond at the intermediate dihydropteridine stage, followed by fission of the polyhydroxy side chain would give xanthopterin (LIV). The feasability of this sequence was admirably demonstrated by Wood and Neilson[73] who synthesised the pyrimidine (LV), ring closed it intramolecularly, and detected 2,4,6-trihydroxy pteridine (LVI in the reaction mixture.

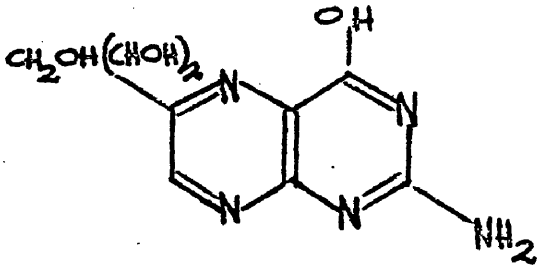
Wood also pointed out[73] that reduction of the keto-group in (LII, R = OH) would give the riboflavin intermediate (XXXVIII), R = D-ribityl).



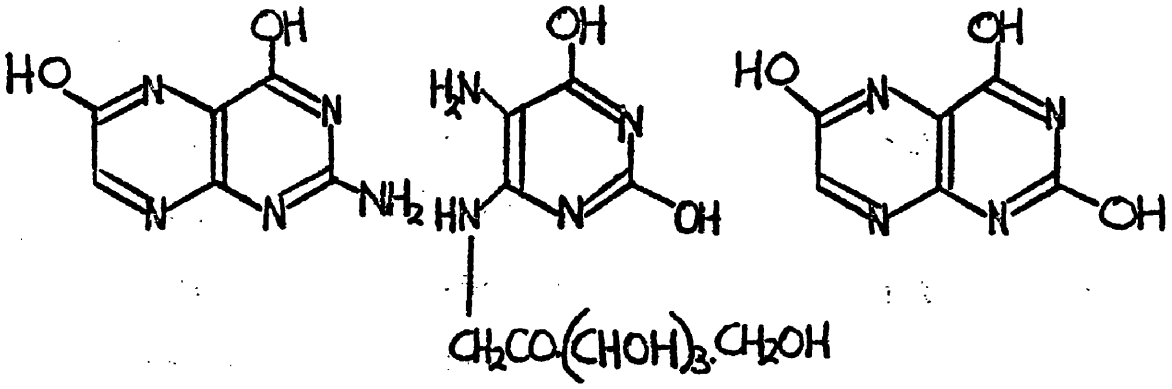
LI



LII

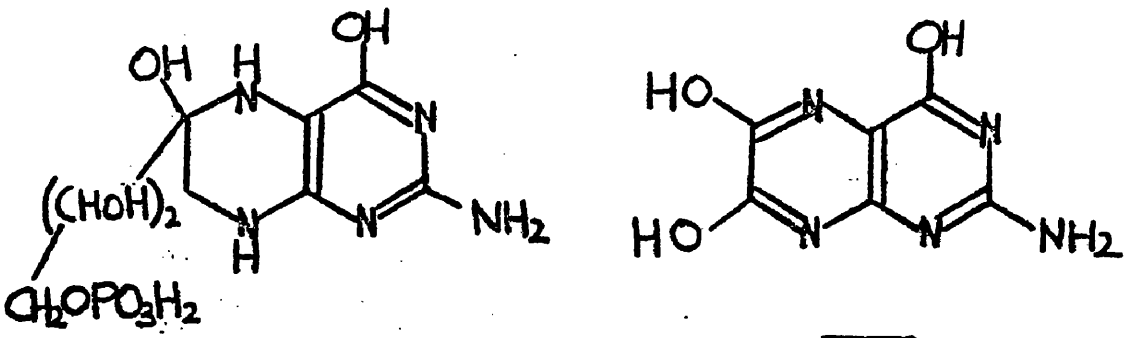


III



LIV LV LVI

The theory of Weygand[74] was very similar and stated that the possibility of the 5'-phosphate of (LI) undergoing ring opening and Amadori rearrangement to give the 5'-phosphate of (LII, R = NH_2) which underwent intramolecular ring-closure to give (LVII) seemed reasonable. He postulated that this pteridine would then lose its side-chain and undergo oxidation to give xanthopterin. Addition of a molecule of water and further oxidation would then give leucopterin (LVIII).



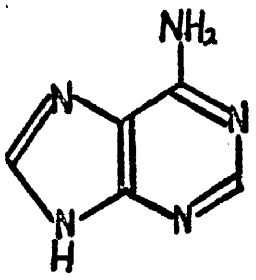
LVII LVIII

Weygand's reasons for postulating this route are interesting. If 3-4 day-old cocoons of Pieris brassicae

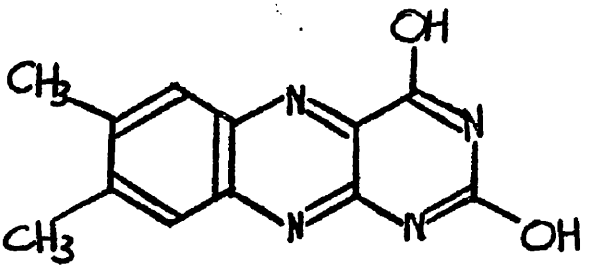
are inoculated with glucose [2-^{14}C] or ribose[1-^{14}C] then a 78 and 64% respectively recovery of label is found in carbon atoms 6 and 7 of the resulting leucopterin.[74] These results are certainly in keeping with what would be expected if the above sequence of reactions were the operative one.

Appendix on Ring-Opening.

Adenine (LIX) exclusively labelled in position 8 with ^{14}C gives, using E.ashbyii, riboflavin which contains only 9% of the original activity of the purine. This 9% was found to be distributed in the manner 45% in the isoalloxazine nucleus and 55% in the ribityl side chain by conversion of riboflavin to lumichrome (LX). This result was taken to indicate "loss" of carbon 8 in the transformation of purines to riboflavin.[39]



LIX

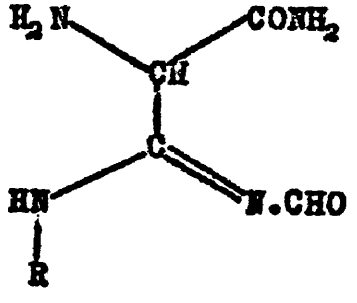


LX

Theoretically then if randomly labelled adenine is added one would expect, since each carbon atom can be taken as possessing 20% of the total activity, that 91% of 20% i.e.

18.2% of the total activity would be "lost". The remainder of carbon-8's share of the total activity 1.8% would be redistributed as .81% to the isoalloxazine nucleus of riboflavin, and .99% to the ribityl side chain. This would give a final side chain activity of 1.21% $\left(\frac{.99}{80 + 1.81} \times 100\right)$ and a final nucleus activity of 98.79% $\left(\frac{80 + .81}{80 + 1.8} \times 100\right)$ of the total remaining activity in the riboflavin produced. McNutt, however, found[40] that the side chain has in fact 6% of the remaining activity and the isoalloxazine nucleus only 94% indicating some contribution of labelled carbon to the side chain from some position other than position 8.

The only position which could possibly donate labelled carbon without complete rupture of the adenine molecule is position 2, by means of an open-chain compound such as (LXI), so it is reasonable to assume that if there is any such contribution it comes from there.



LXI

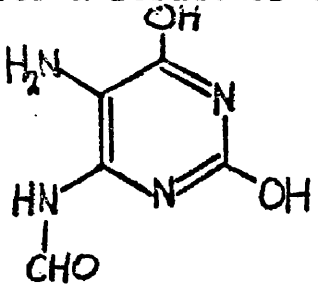
If one assumes that all the labelled carbon "lost" from position 2 is "regained" by the side chain then one finds that carbon 2 must contribute 3.91% (If x is the % of carbon 2

remaining in the pyrimidine ring, then x = [.94 x 81.8]-[60 + .81] or x = 16.09%) of the total original activity of the adenine to the riboflavin side chain, which is 19.55% of its own original activity. On this evidence McNutt states that the pyrimidine ring goes intact,[40] the earlier results which caused him some doubt when submitted to the above calculation require a label transfer from carbon 2 as high as 76% of its original activity.[39] More recently Plaut[86] studying the incorporation of adenine specifically labelled in position 8 with ^{14}C reported that there was considerable incorporation of label in the resulting riboflavin.

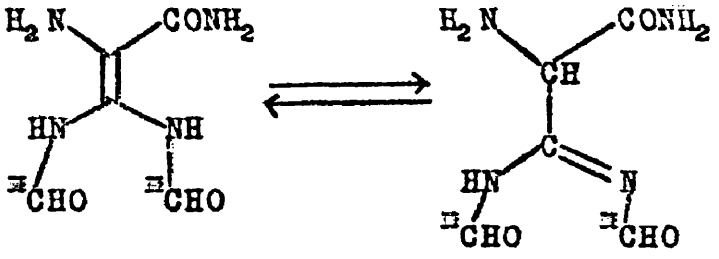
In support of McNutts conclusion that no ring opening takes place two considerations are outstanding. Firstly if carbon-2 of the purine does contribute to any great extent to the side chain in riboflavin then this would amount to contribution of formate to the side chain, and experiments using labelled formate show that most of the formate contribution to riboflavin can be accounted for in the nucleus.[33,78] In the second place the amount of label in the individual carbon atoms in the pyrimidine ring of riboflavin after incorporation of randomly labelled adenine shows no deficiency of label in carbon-2 compared with carbons 4,5 and 6.[40]

In support of the possible opening two other results spring to mind. Incorporation studies using guanosine equally labelled with ^{14}C in the nucleus and the sugar side chain

gave riboflavin in which more than 90% of the activity was in the isoalloxazine nucleus.[69] This is exactly the result one would expect if the mechanism of transformation was one which involved removal of sugar giving an intermediate of type (LXII), which then opened in the pyrimidine ring to give (LXIII). Rotation about the central carbon-carbon bond would in effect interchange the carbons marked thus*. Finally incorporation of a new sugare would give an overall effect similar to that of the randomly labelled purine free base which as we know in the case of adenine gives a result of this order.[40]



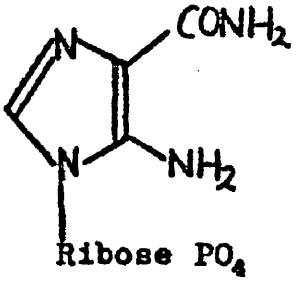
LXII



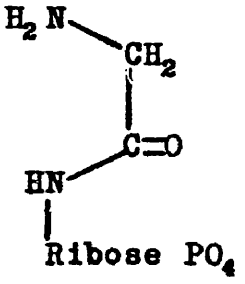
LXIII

The other interesting discovery which lends weight to such a mechanism is reported by Goodwin et al.[42] These workers found that aminopterin inhibited growth or purine formation as usual but to their surprise it promoted flavinogenesis. As it seemed well-established that purines were transformed into riboflavin it was expected that anything which inhibited growth would also inhibit flavinogenesis. The only obvious explanation of this strange result is that E.ashbyii can transform the inhibited puring intermediate into a riboflavin intermediate. As aminopterin inhibits by blocking the insertion of one-carbon

units the obvious purine intermediates that would be blocked are either (LXIV) or (LXV),[76] and both of these suggest a pyrimidine ring-open intermediate in flavinogenesis.



LXIV



LXV

It seems to the author that further study of possible pyrimidine ring opening in the route purine to riboflavin is merited on the above evidence. This is, however, not considered further in this thesis.

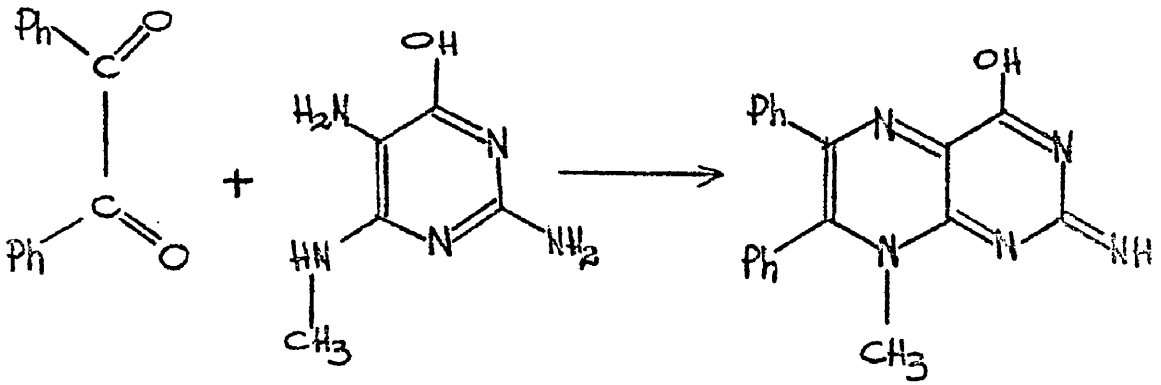
PART I

The Synthesis of Riboflavin 2-Imine and Related isoAlloxazine 2-Imines.

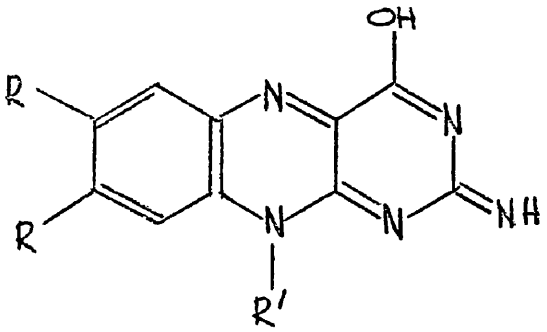
Theoretical

Recently two syntheses of an isoalloxazine 2-imine (LXVI, R = R' = CH_3) have been reported[29,30] and these have been discussed earlier in this thesis. In view of the low yields in these reactions, together with the observation[47] that many unsuccessful attempts have been made to prepare riboflavin 2-imine, it appeared unlikely that standard isoalloxazine syntheses would be successful for the synthesis of this flavin.

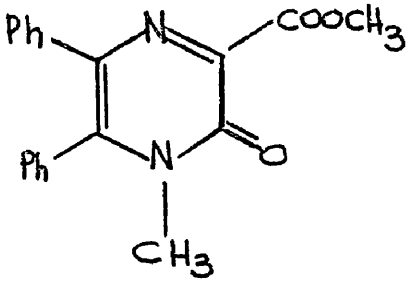
Two recent pteridine syntheses are of interest when considering a new synthetic approach to the isoalloxazines. In the first of these Fidler and Wood[77] reported the synthesis of 2-imino-8-substituted-pteridines by condensation of αβ-dicarbonyl compounds with 5-amino-4-alkyl (or aryl) amino-pyrimidines (LXVII), e.g.



LXVII LXVIII



LXVI

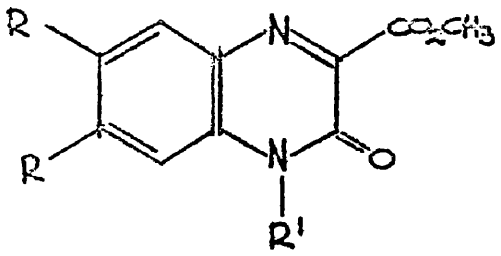


LXIX

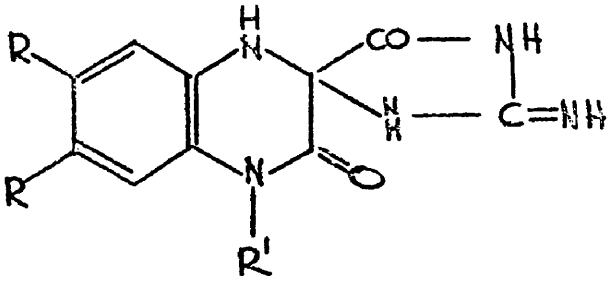
The iminopteridine (LXVIII) was identical with the product obtained in a second synthesis[78] which involved fusion of the pyrazine ester (LXIX) with guanidine carbonate.

Adaptation of the first of these methods to the synthesis of riboflavin 2-imine was premature as the necessary pyrimidine intermediate, 2,5-diamino-4-D-ribitylamino-6-hydroxy pyrimidine, was at that time unattainable. It is of interest that this compound was later synthesised in this department by Larder[79] who used a method similar to that reported later in this thesis for the synthesis of the corresponding 2-hydroxy pyrimidine.

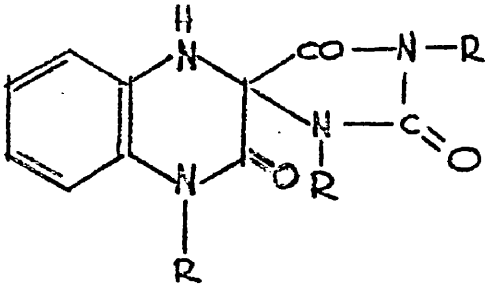
It seemed practical however to adapt the second of the above pteridine synthesis, and prior to the work reported in this thesis Hill[80] had investigated this type of condensation with considerable success using the quinoxaline ester (LXX, R = H, R' = CH_3).



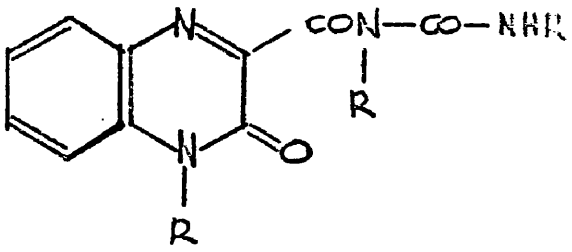
LXX



LXXI



LXXII

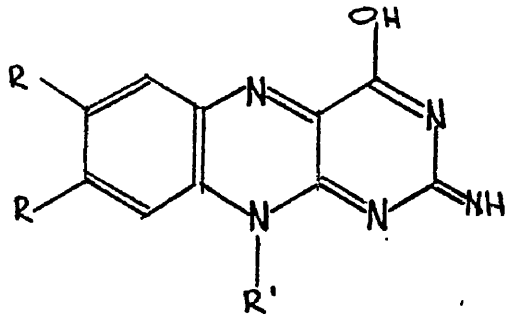


LXXIII

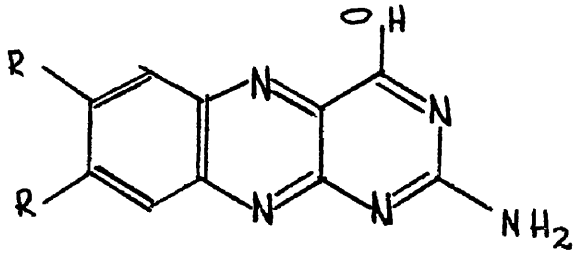
He reported that condensation of this ester with guanidine in the presence of sodium methoxide gave the tricyclic spiro compound (LXXI, R = H, R' = CH_3). This he confirmed by comparison of the U.V. absorption spectrum of his product with that of a similar spiro compound (LXXII, R = CH_3, R' = R'' = H) obtained by Clark-Lewis[81] from treatment of the carboxyureide (LXXIII, R = CH_3, R' = R'' = H) with anhydrous potassium carbonate in dry acetone at an elevated temperature. Hill also condensed the quinoxaline ester (LXX, R = H, R' = CH_3) with urea and produced a compound

which on methylation with methyl iodide gave the spiro compound (LXXII, R = R' = R'' = CH_3). This proved to be identical with an authentic specimen produced by methylation of Clark-Lewis' spiro compound (LXXII, R = CH_3, R' = R'' = H).[81]

Hill also reported[80] that more prolonged reaction of the ester (LXX, R = H, R' = CH_3) with guanidine in sodium propoxide in the dark gave a mixture of the isoalloxazine 2--imine (LXXIV, R = H, R' = CH_3) and 2-amino-4-hydroxybenzo[g] pteridine (LXXV, R = H). When the reaction was repeated in daylight, the sole product was the amino-alloxazine (LXXV, R = H).



LXXIV

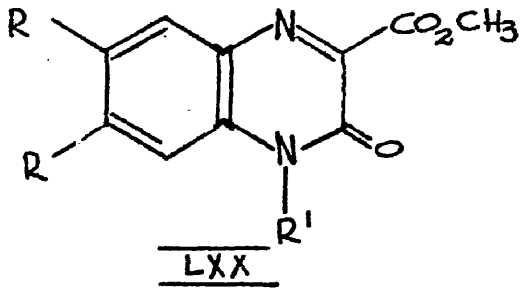


LXXV

It was therefore decided that condensation of the ester (LXX, R = H, R' = CH_3) with guanidine in the dark should be reinvestigated to confirm the findings[80] of Hill and to explain, if possible, the unexpected result.

The methyl ester (LXX, R = H, R' = CH_3) was prepared by N-methylation of the corresponding hydroxy ester.[80] It was then condensed with guanidine in sodium propoxide in the dark and in an atmosphere of nitrogen. After refluxing

for three days the reaction was stopped and the reaction mixture allowed to come to room temperature. This gave a bright orange precipitate consisting of two main products. Attempted separation by column chromatography using cellulose powder was not completely successful, but the products were separated by making use of the different solubilities of their hydrochlorides. By this means the products were shown to be the isoalloxazine 2-imine (LXXIV, R = H, R' = CH_3), and the aminoalloxazine (LXXV, R = H) as reported by Hill.[80]

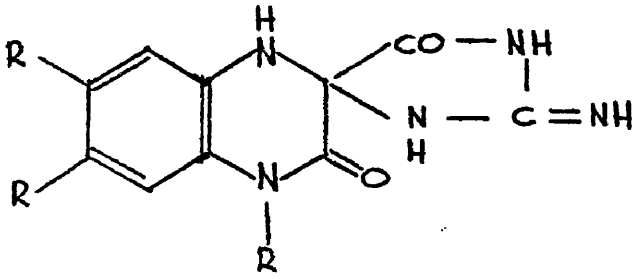


LXX

An analogous synthesis of lumiflavin 2-imine (LXXIV, R = R' = CH_3) was now attempted as this would enable direct comparison to be made of the product of this new isoalloxazine synthesis with that of established methods.[29,30]

Lumiflavin obtained by irradiation of riboflavin[82] was degraded using sodium hydroxide to give 3,4-dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylic acid.[83] Treatment of this acid with excess diazomethane gave the methyl ester (LXX, R = R' = CH_3) required for condensation reactions. This quinoxaline ester was treated with guanidine in sodium propoxide

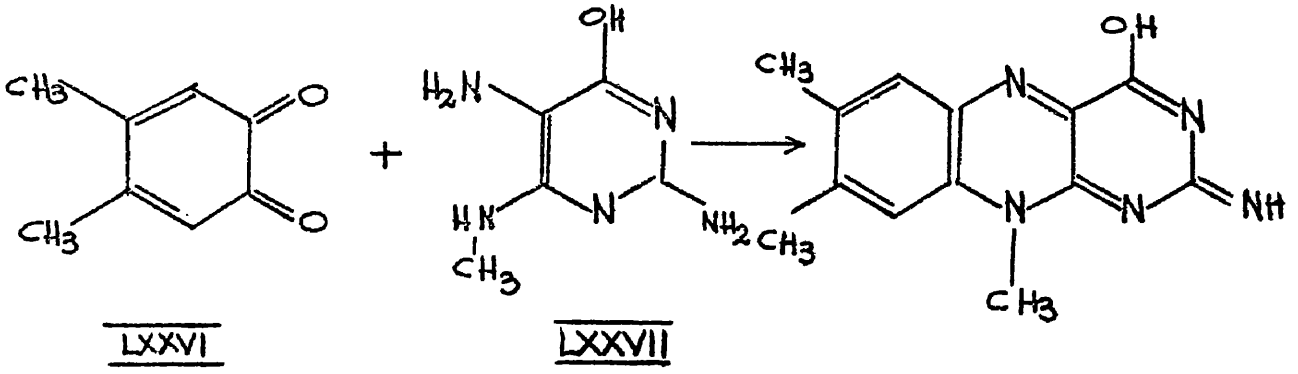
and the mixture was incubated at 25°. Removal of the alcohol gave a product which on treatment with hydrochloric acid gave the hydrochloride of the spiro compound (LXXI, R = R' = CH_3). The free base was obtained by treatment of the hydrochloride with sodium hydrogen carbonate in aqueous solution. The ultraviolet spectrum of this product (see Table 1) was very similar to that of the spiro compound reported by Hill.[80]



LXXI

Condensation of the methyl ester (LXX, R = R' = CH_3) with guanidine in sodium propoxide was therefore repeated under more drastic conditions by refluxing for three days in the dark and in an oxygen-free atmosphere. This method gave as the main product a yellow pigment which was shown to contain 2,10-dihydro-4-hydroxy-2-imino-7,8,10-trimethylbenzo[g]pteridine (LXXIV, R = R' = CH_3, "lumiflavin 2-imine") both by paper chromatography and by ultraviolet absorption spectroscopy. The yield could be improved only slightly by refluxing the yellow pigment in methanol, and no satisfactory method of separating lumiflavin 2-imine from this yellow pigment could be found.

It seemed desirable at this stage to obtain larger quantities of lumiflavin 2-imine, and it was decided to attempt a new synthesis based on the first pteridine synthesis discussed at the beginning of this section. The proposed reaction would involve condensation of 3,4-dimethyl-o-benzoquinone (LXXVI) with 2,5-diamino-4-hydroxy-6-methylaminopyrimidine (LXXVII).



LXXVI LXXVII

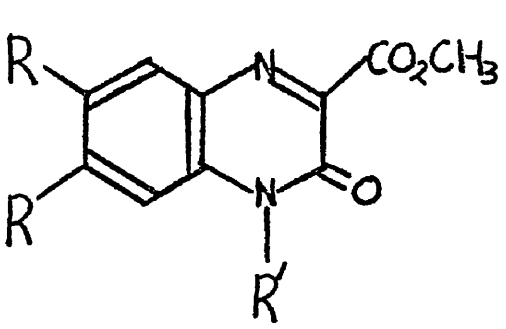
3,4-Dimethyl-o-benzoquinone was prepared by standard procedures[84,85,86] and converted to the stable yellow dimer by treatment with glacial acetic acid. This dimerisation was carried out because Bardos et al.[84] have reported that the dimer gives a better yield of alloxazines when condensed with unsubstituted diaminopyrimidines than does the monomeric compound. The dimeric quinone was reacted with 2,5-diamino-4-hydroxy-6--methylaminopyrimidine[77] (LXXVII) in alkaline solution and the green product seemed to be lumiflavin 2-imine from its ultraviolet absorption spectrum, and when recrystallised from hydrochloric acid gave orange needles of a hydrochloride which was identical with the hydrochloride of lumiflavin 2-imine prepared by standard procedures (The author is indebted to Dr. P. Hemmerich of the

University of Basle for carrying out this comparison).

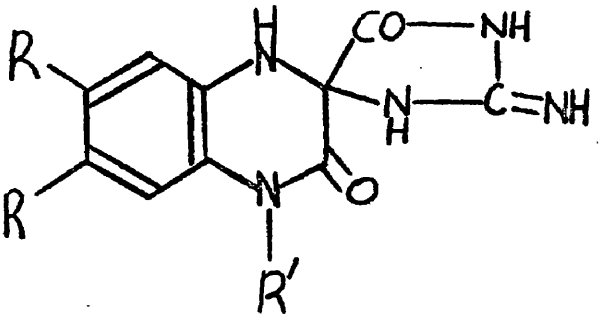
The most striking property of lumiflavin 2-imine compared with other isoalloxazine 2-imines is that it is extremely insoluble in alkali. During the condensation of the quinoxaline ester (LXX, R = R' = CH_3) with guanidine in sodium propoxide considerable difficulty was encountered with "bumping" due to a heavy precipitation during the reaction. It is just possible that at some stage in this reaction an intermediate compound is produced which is also very insoluble in alkali and that this insolubility of a vital intermediate is the reason for the low yield in the reaction.

In the hope that riboflavin 2-imine (LXXIV, R = CH_3, R' = D-ribityl) would be produced in a better yield, an analogous synthesis was carried out starting from methyl 3,4-dihydro-6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline-2-carboxylate (LXX, R = CH_3, R' = D - ribityl). This compound was prepared by esterification of the corresponding acid[87] which is readily obtained from riboflavin by treatment with dilute sodium hydroxide. The esterification proceeded smoothly using diazomethane or more easily by heating the acid with methanol.

This ester (LXX, R = CH_3, R' = D-ribityl) with guanidine in the presence of sodium propoxide gave a high yield of 2,3,4,5, 1',2',3',4'-octahydro-2-imino-6',7'-dimethyl-

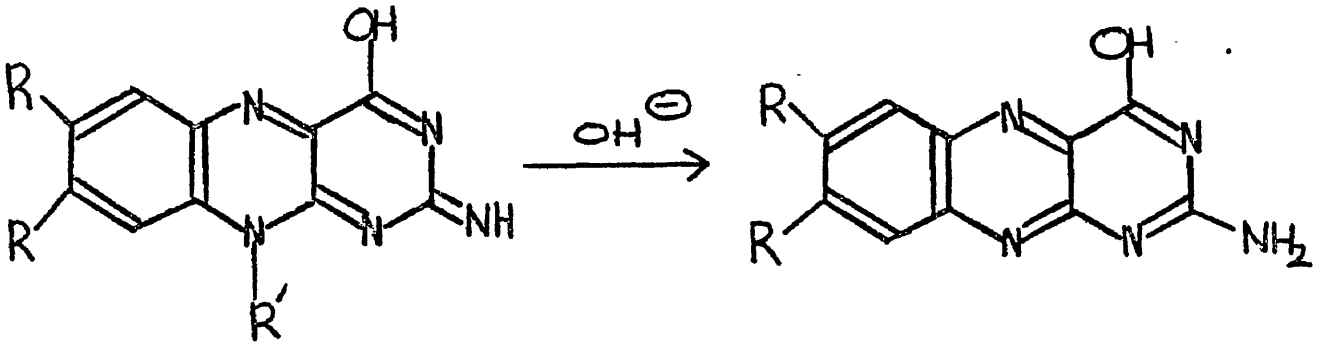


LXX



LXXI

-3',5-dioxo-4'-D-ribitylglyoxaline-4-spiro-2'-quinoxaline (LXXI, R = CH_3, R' = D-ribityl) which was readily converted to its crystalline hydrochloride. More prolonged reaction in daylight and in the presence of air gave 2-amino-4-hydroxy-7,8-dimethylbenzo[g]pteridine (LXXV, R = CH_3; "2-amino lumichrome"), apparently identical with the material prepared by Bardos, Olsen, and Enkoji.[84] A similar reaction carried out under nitrogen and in the dark gave a mixture of products including a large proportion of 2,10-dihydro-4-hydroxy-2-imino-7,8-dimethyl-10-D-ribitylbenzo[g]pteridine (LXXIV, R = CH_3, R' = D-ribityl; "riboflavin 2-imine"). Isolation of pure riboflavin 2-imine was achieved, with difficulty, by chromatography on "Florisil" columns, or alternatively by repeated precipitation from acid. These procedures were carried out in the dark as riboflavin 2-imine readily underwent photolysis.

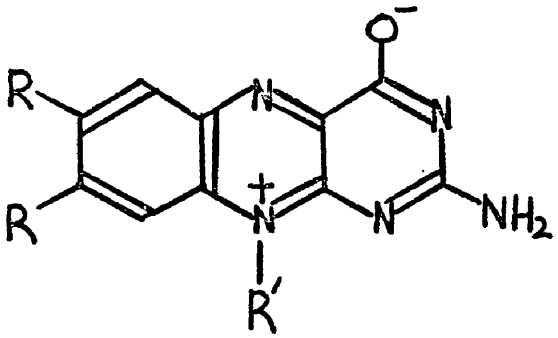


LXXIV LXXV

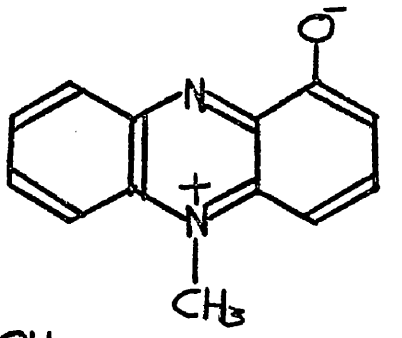
The isoalloxazine 2-imines (LXXIV) when treated with dilute alkali in the presence of air and in the dark, readily lose the 10-alkyl substituent with the formation of 2-amino-alloxazine derivatives (LXXV). This behaviour is in distinct contrast with that of isoalloxazines such as riboflavin[87] and lumiflavin[83] which under similar conditions undergo ring cleavage to quinoxaline derivatives, and it may have some bearing on the biological transfer of C_1-C_5 fragments. One further interesting observation is that in the case of riboflavin 2-imine in cold dilute alkali the product was a mixture of 2-aminolumichrome and lumiflavin 2-imine. Since lumiflavin 2-imine is not converted to 2-aminolumichrome in cold alkali owing to its insolubility there must be two distinct degradations of riboflavin 2-imine, at least in the cold reaction, similar to the two distinct degradations[82] which

riboflavin undergoes on photolysis in alkaline solution to give lumiflavin and lumichrome.

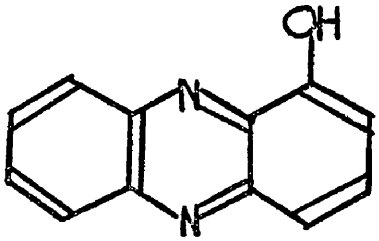
For isoalloxazines and isoalloxazine 2-imines (LXXIV) there is the possibility of resonance involving dipolar forms such as (LXXVIII). The insolubility of the imines in chloroform and other non-polar solvents makes it likely that the contribution of such ionic forms to the resonance hybrid is much greater than with isoalloxazines such as lumiflavin which readily dissolve in chloroform. The existence of such contributing structures may explain the ease with which 10-alkyl substituents may be cleaved from isoalloxazine 2-imines (LXXIV). The bacterial pigment, pyocyanine (LXXIX), which has an analogous dipolar structure,[88] is similarly demethylated[89] in alkaline solution to 1-hydroxyphenazine (LXXX).



LXXVIII



LXXIX

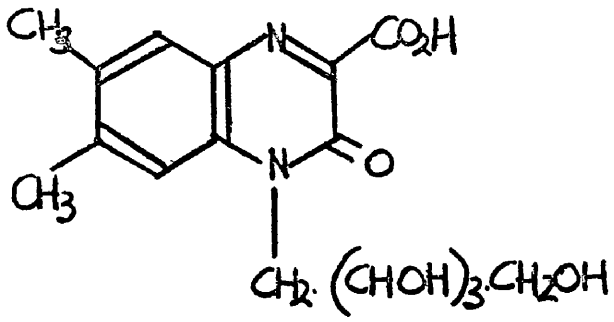


LXXX

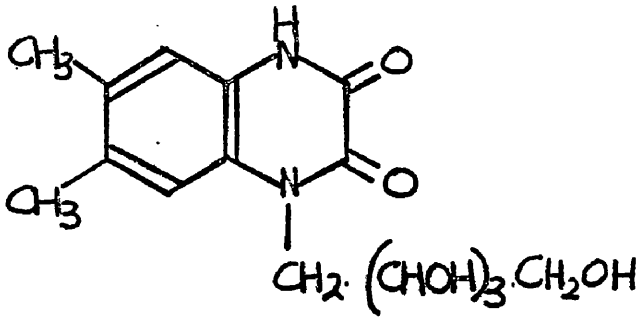
Irradiation of riboflavin 2-imine (LXXIV, R = CH_3, R' = D-ribityl) with ultraviolet light gave products analogous to those obtained on photolysis of riboflavin.[82] Thus in acid solution 2-aminolumichrome (LXXV, R = CH_3) was produced, and in alkaline solution a mixture of lumiflavin 2-imine (LXXIV, R = R' = CH_3), 2-aminolumichrome (LXXV, R = CH_3) and a third, unidentified, product which on the basis of its ultraviolet absorption spectrum was probably a quinoxaline. Irradiation of lumiflavin 2-imine (LXXIV, R = R' = CH_3) in acid solution also gave 2-aminolumichrome (LXXV, R = CH_3).

In contrast to the above degradations brought about by the action of light or alkali, the isoalloxazines (LXXIV) were apparently stable to heat. Thus when an aqueous solution of an imine was heated no degradation was observed.

During condensations using the quinoxaline ester (LXX, R = CH_3, R' = D-ribityl) or its parent carboxylic acid (LXXXI) a blue-fluorescing compound persisted on paper chromatograms. This compound was shown by paper chromatography to be present in the reaction mixture of the riboflavin 2-imine condensation, and also in that of the ribityl spiro compound (LXXI, R = CH_3, R' = D-ribityl). It was likewise produced by photolysis of the acid (LXXXI) in either acid or alkali.



LXXXI



LXXXII

A small quantity of the blue fluorescing compound was obtained from the riboflavin 2-imine reaction by means of chromatography on a cellulose powder column. This product was shown to be identical both by paper chromatography and elementary analysis with a quinoxaline (LXXXII) which Miles, Smyrniotis, and Stadtman[90] obtained from bacterial decomposition of riboflavin. Miles et al. reported, without experimental detail,[90] that the acid (LXXXI) could be converted to the quinoxaline (LXXXII) by the action of hydrogen peroxide in acetic acid. An experiment using the acid (LXXXI), hydrogen peroxide, and acetic acid was therefore performed and gave a product which was the same as that isolated in the above reactions. On this evidence it seems likely that the blue fluorescing compound has the structure (LXXXII). The literature description[90] of the light-absorbing properties of this compound is a little confused.

Hiles *et al.*[90] give figures in the text of their paper which agree with those determined in the course of the present work (see Table 1). In the same paper, however, a graph is given which is obviously in error, the curves at pH1 and pH13 having been labelled in the reverse manner.

TABLE I.

Compound	R_f (A)	R_f (B)	$\lambda_{max.}$ (ε in parentheses) in H_2O at pH given	
Spiran (LXXI)				
R = CH_3, R' = CH_3	0.60	-	226(28,000), 306(4600)	pH13
			218(31,100), 241(21,800), 340(11,300), 410(7,500)	pH1
R = CH_3, R' = D-ribityl	0.24	-	230[a](18,900), 306(4,500)	pH7
			220(31,500), 240[a](22,700), 342(11,300), 412(7,200)	pH1
Compounds (LXXV)				
R = H	0.46	0.14	218(28,800), 262(44,500), 333(7,000), 416(9,000)	pH13
			212(27,800), 244(23,500), 263(32,900), 345(9,200), 370[a](6,200), 430[a](2,100)	pH1
R = CH_3	0.54	0.03	224(23,600), 264(43,650), 344(5,500), 420(7,400)	pH13
			220(23,900), 266(27,500), 362(8,200)[b]	pH1

TABLE I (contd.)

Compound	R_f (A)	R_f (B)	λmax. (ε in parentheses) in H_2O at pH given	
Compounds (LXXIV)				
R = H, R' = CH_3	0.23	0.36	216(24,500), 264(29,800)	pH1
			358(8,200), 428(11,020)	
R = CH_3, R' = CH_3	0.34	0.23	223(21,100), 268(25,000)	pH1
			384(9,100), 443(10,300)	
R = CH_3, R' = D-ribityl	0.22	0.28	224(27,000), 270(32,100)	pH1
			386(12,300), 442(14,100)	
Riboflavin	0.22	0.39	223(35,500), 267(35,500)	pH1
			376(10,700), 445(11,500)	
Compound (LXXXII)	See experimental		214(49,800), 234[a](12,500)	pH1
			266(4,600), 324(12,500)[c]	
			220(45,000), 239(16,300),	
			322(13,100), 334(14,500), 348[a](9,800)[c]	pH13

[a] Shoulder [b] Bardos et al.,[85] give max. 266(ε,33,900) and 362 mu (10,720).

[c] Miles et al.[90] give 213(46,000), 234(12,100), 263(3,190) and 323 mu(10,700) at pH1, and 222(39,000), 239(14,000), 322(12,200), 334(13,300), and 348 mu[a](8,550) at pH13.

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, 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 mu. Infra-red spectra were determined for Nujol mulls and potassium chloride discs.

3-Hydroxyquinoxaline-2-carboxyureide.[28] - Alloxan-monohydrate (3.7 g.) in water (75 c.c.) was treated with a solution of o-phenylenediamine (2.5 g.) in water (150 c.c.). The mixture was allowed to stand at room temperature for 20 min. and the resulting yellow micro-crystalline solid was collected. Recrystallisation from a large quantity of glacial acetic acid gave the carboxyureide (4.85 g., 90%) as yellow micro-crystals, m.p. 250° (lit.,[28] 250°).

3-Hydroxyquinoxaline-2-carboxylic Acid.[28] - 3-Hydroxyquinoxaline-2-carboxyureide (4.7 g.) was refluxed for 4 hr. in 5N-sodium hydroxide (30 c.c.). The reaction mixture after cooling was acidified to pH4 using 5N-hydrochloric acid.

The precipitated solid was collected, washed with water, and dried. Recrystallisation from water gave the carboxylic acid as yellow prismatic needles (3.2 g., 80%), m.p. 268° (lit.,[28] 268°).

Methyl 3-Hydroxyquinoxaline-2-carboxylate[80]. - 3-Hydroxyquinoxaline-2-carboxylic acid (1.6 g.) suspended in dry methanol (100 c.c.) saturated with dry hydrogen chloride was refluxed for 2 hr. Refrigeration gave the methyl ester (1.48 g., 86%) which recrystallised from methanol as prisms, m.p. 222° (lit.,[80] 222°).

Methyl 3,4-Dihydro-4-methyl-3-oxoquinoxaline-2--carboxylate.[80] - Methyl 3-hydroxyquinoxaline-2-carboxylate (12 g.), methyl iodide (20 g.), and anhydrous potassium carbonate (60 g.) were refluxed in dry acetone (180 c.c.) for 24 hr. The mixture was then cooled, water was added, and the whole extracted with chloroform. Evaporation of the dried chloroform extract, and recrystallisation of the residue from methanol gave the N-methyl ester (10 g., 78%) as rosettes of needles, m.p. 126° (lit.,[28,80] 126°).

2,10-Dihydro-4-hydroxy-2-imino-10-methyl benzo [g]pteridine. - A solution from sodium (0.347 g.) in dry propan-1-ol (40 c.c.) was added to a warm solution of dry

guanidine hydrochloride (1.49 g.) in dry propan-1-ol (50 c.c.). The mixture was added to a hot solution of methyl 3,4-dihydro-4--methyl-3-oxoquinoxaline-2-carboxylate (1 g.) in propan-1-ol (50 c.c.), and thenrefluxed for 72 hr. in the dark in an oxygen-free nitrogen atmosphere. The cooled reaction mixture yielded a bright orange precipitate (1.6 g.) readily separated by paper chromatography into 2-amino-4-hydroxybenzo[g]pteridine and the desired isoalloxazine-2-imine. Chromatography of a larger sample (0.3 g.) on a column of cellulose powder (Whatman No.1; 70 g.) with butan-1-ol-water-acetic acid (63:27:10) as solvent did not, however, separate the two components completely. The remainder of the crude product (1.3 g.) was dissolved in the minimum quantity of hot 5N-hydrochloric acid. The hydrochloride of 2-amino-4-hydroxybenzo[g]pteridine was filtered off, and the filtrate was diluted with ethanol and evaporated to dryness *in vacuo*. The residue, when shaken with dry ethanol (100 c.c.) gave a bright orange powder (100 mg.). Recrystallisation from ethanol gave the bright yellow *isoalloxazine-2-imine*, m.p. $>350°$ (Found: C,53.3; H,4.0. $C_{11}H_9ON_5.H_2O$ requires C,53.9; H,4.5%).

Lumiflavin.[37] - Riboflavin (4.7 g.) was irradiated (16 hr.) in 2N-sodium hydroxide (2000 c.c.) in an open trough (liquid depth 1.5 in.) equipped with a mechanical stirrer, using a U.V. lamp.(Hanovia, 380 watt.) at a distance of 15 cm.

The irradiated solution was acidified using 5N-sulphuric acid and extracted with chloroform until the chloroform layer was colourless. The chloroform extract, after drying over sodium sulphate, was evaporated to dryness *in vacuo* to yield crude lumiflavin (2.1 g.; 56.5%). U.V. spectrum in 0.2N-sodium hydroxide had maxima at 218,266, 350 and 420 mu.

3,4-Dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylic Acid.[83] - A solution of crude lumiflavin (10.4 g.) in 2N-sodium hydroxide was refluxed for 1 hr. After filtration the solution was acidified with concentrated hydrochloric acid and extracted with chloroform using a continuous solvent extractor. The dried chloroform extract was reduced to dryness to give the carboxylic acid (4.1 g., 42.7%) which readily recrystallised from methanol to give orange minute needles, m.p. 202-204° (lit.[40], 213°).

Methyl 3,4-Dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylate. - 3,4-Dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylic acid (2.8 g.) was esterified with diazomethane (8 equivs.). Recrystallisation from methanol gave the methyl ester (1.95 g., 66%) as yellow plates, m.p. 169-170° (Found: C,63.8; H,5.9; N,11.4. $C_{13}H_{14}O_3N_2$ requires C,63.4; H,5.7; N,11.4%).

2,3,4,5,1',2',3',4'-Octahydro-2-imino-4',6',7'--trimethyl-3',5-dioxoglyoxaline-4-spiro-2'-quinoxaline. - To a solution from sodium (0.77 g.) in dry propan-1-ol (12.5 c.c.) was added dry powdered guanidine hydrochloride (.338 g.) in propan-1-ol (12.5 c.c.). The mixture, after filtration, was added to a solution of methyl 3,4-dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylate (0.25 g.) in dry propan-1-ol (12.5 c.c.) and kept at 25° for 20 hr. The volume of the solution was reduced, the cream-coloured precipitate collected and recrystallised from 2N-hydrochloric acid to give the hydrochloride of the spiro compound (0.12 g.) as yellow needles, m.p. 267-270° (Found: C,50.1; H,5.4; N,22.3. $C_{13}H_{18}O_2N_6$, HCl requires C,50.4; H,5.2; N,22.6%).

The free base was prepared by dissolving the hydrochloride (0.043 g.) in water and adjusting the pH to 7 by the addition of solid sodium hydrogen carbonate. The pale cream precipitate (0.02 g.) was collected, washed with hot water, and dried in vacuo to give the base as a hygroscopic powder, m.p. 275° (Found: C,57.1; H,5.5. $C_{13}H_{18}O_2N_6$ requires C,57.1; H,5.5%).

2-Phenylazo-4,5-dimethyl Phenol. -[85,86,91] 3,4-Xylen--1-ol (14 g.) and 10% sodium hydroxide (93 c.c.) were stirred until the solution became homogeneous.

Aniline (12.5 c.c.) and ice (150 g.) were treated with concentrated hydrochloric acid (41 c.c.; sp.gr. 1.16).

A solution of sodium nitrite (10 g.) in water (20 c.c.) was then added slowly and the mixture was stirred until diazotisation was complete.

To the alkaline solution of the phenol cracked ice (100 g.) was added followed by the diazonium salt solution. The solution was then stirred for 30 min. while the temperature was kept below 10°, and then left standing for a further 3 hr. at 7-10°. The orange precipitate was collected, washed with water (100 c.c.), stirred for 10 min. with 10% sodium hydroxide (300 c.c.), recollected, washed with water, and recrystallised from ethanol to give 2-phenylazo-4,5-dimethyl phenol (17 g.) as deep red needles, m.p. 129-131°. (lit.,[85] 130°).

2-Amino-4,5-dimethyl Phenol.[84] - To a suspension of 2-phenylazo-4,5-dimethyl phenol (17 g.) in ethanol (250 c.c.) was added Raney nickel (2 g.), and the mixture was hydrogenated in a Cook hydrogenation apparatus at an initial hydrogen pressure of 4 atmospheres. When uptake of hydrogen ceased the catalyst was removed by filtration and the alcohol by distillation *in vacuo*. The residue was washed with cold toluene (100 c.c.) and recrystallised from hot toluene to give 2-amino-4,5-dimethyl phenol (5 g.), m.p. 174° (lit.,[84] 173°-175°).

Monomer of 4,5-Dimethyl-o-benzoquinone.[84] - 2-Amino--4,5-dimethyl phenol (5 g.) was dissolved in water (350 c.c.) and

concentrated sulphuric acid (9 c.c.). This solution was poured rapidly, with stirring, into a solution of potassium dichromate (8 g.) in water (350 c.c.). The solution was extracted immediately with chloroform (150 c.c.) and the chloroform layer was dried over anhydrous sodium sulphate. The clear solution was concentrated *in vacuo* to give the monomeric 4,5-dimethyl-*o*-benzoquinone (2 g.) as deep red crystals after washing with cold ether, m.p. 95° (Lit.,[84] 95°).

Dimer of 4,5-*Dimethyl*-o-*benzoquinone*.[84] Monomeric 4,5-dimethyl-*o*-benzoquinone (2 g.) was dissolved with heating in glacial acetic acid (11 c.c.) and water (65 c.c.) was then added. After standing for 8 days at room temperature the precipitated dimer (1 g.) was collected, washed with water and dried, m.p. 168°. Recrystallisation from 2-propanol gave dimeric 4,5-dimethyl-*o*-benzoquinone (0.39 g.) as yellow crystals, m.p. 173-175° (Lit.,[84] 178-180°).

2-*Amino*-4,6-*dihydroxypyrimidine*.[80] Dry powdered guanidine hydrochloride (200 g.) and diethyl malonate (320 g.) were added to a solution from sodium (75 g.) in ethanol (1.5 l.). The mixture was refluxed for 2 hr. at the end of which the best part of the ethanol was removed by distillation *in vacuo*. The resulting solid was treated with water (2 l.) and the solution acidified by the addition of glacial acetic acid. The product was collected, and dried at

115°, to give the pyrimidine as an off-white powder (230 g.), m.p. > 300°.

2-Amino-4,6-dichloropyrimidine.[80,92] - 2-Amino-4,6-dihydroxypyrimidine (100 g.) was refluxed with phosphoryl chloride (400 c.c.) and diethylaniline (100 c.c.) until complete solution. The dark viscous solution was poured into crushed ice (1500 g.). The resulting brown solid was collected and dried. 2-Amino-4,6-dichloropyrimidine was obtained as lustrous white plates (35 g.), m.p.221° (lit.[80], 221°), by heating the brown solid at 165°/15 mm. in a large sublimation apparatus.

2-Amino-4-chloro-6-hydroxypyrimidine.[80,93] 2-Amino--4,6-dichloropyrimidine (30 g.) was refluxed for 8 hr. in N-sodium hydroxide solution (370 c.c.). The solution was filtered, cooled, and acidified with glacial acetic acid to give the 2-amino-4-chloro-6-hydroxypyrimidine as a white powder (24.8 g.), m.p. 260-262° (lit.[93], 261°).

2-Amino-4-hydroxy-6-methylaminopyrimidine.[77] - 2-Amino-4-chloro-6-hydroxypyrimidine (8 g.) and ethanolic methylamine (33% w/w; 25 c.c.) were heated at 120° for 4 hr. The solution was cooled, and the precipitate was collected and dissolved in hot dilute hydrochloric acid (30 c.c.). The acid solution was treated with charcoal, and sodium hydrogen carbonate was added to give 2-amino-4-hydroxy-6-methylamino-

pyrimidine. Recrystallisation from ethanol gave light brown plates (2 g.), m.p. 255-257°. (lit.[77], 255-257°).

2-Amino-4-hydroxy-6-methylamino-5-nitrosopyrimidine.[77] - 2-Amino-4-hydroxy-6-methylaminopyrimidine (2 g.) in 2N-hydrochloric acid (35 c.c.) was treated dropwise at 0° with sodium nitrite (1.6 g.) in water (20 c.c.). After being chilled overnight the pink precipitate was collected, and recrystallised from water to give the 5-nitrosopyrimidine (1.29 g.) as orange needles, m.p. $>$ 300°.

2,5-Diamino-4-hydroxy-6-methylaminopyrimidine.[77] - 2-Amino-4-hydroxy-6-methylamino-5-nitrosopyrimidine (0.5 g.) in hot sodium hydroxide solution (0.72 g. in 8 c.c. water) was heated to 80°, and a vigorous stream of nitrogen was bubbled through the solution. Sodium dithionite (2 g.) was added during 5 min., the colour of the solution changing from deep red to yellow. Heating under nitrogen was continued for a further 20 min., concentrated hydrochloric acid was added to pH9.5 and the solution was chilled. The rather unstable 2,5-diamino-4-hydroxy-6-methylaminopyrimidine (0.4 g.) was collected, m.p. 204-210° (lit.,[77] 204-210°).

2,10-Dihydro-4-hydroxy-2-imino-7,8,10-trimethyl benzo[g]pteridine. ("Lumiflavin 2-imine"). -

(a) To a solution of dimeric 3,4-dimethyl-o-benzoquinone (0.32 g.) in water (10 c.c.) and 10% aqueous sodium hydroxide

(1c.c.) was added a similar solution of 2,5-diamino-4-hydroxy--6-methylaminopyrimidine (0.4 g.). The mixture was kept at room temperature for 3 days in a stoppered flask. The resulting precipitate was collected, shaken with N-sodium hydroxide (25 c.c.) in which the isoalloxazine 2-imine is insoluble, and finally refiltered and washed with ether. Recrystallisation from the minimum quantity of 2N-hydrochloric acid gave the hydrochloride (0.06 g.) of the benzopteridine as orange needles, m.p. $>$ 360° (Found: C,53.1; H,4.6; N,24.2. $C_{13}H_{13}ON_5.HCl$ requires C,53.5; H,4.8; N,24.0%).

(b) To a solution from sodium (0.3 g.) in dry propan-1-ol (50 c.c.) was added dry powdered guanidine hydrochloride (1.24 g.) in propan-1-ol (50 c.c.). The mixture, after filtration, was added to a solution of methyl 3,4-dihydro-4,6,7-trimethyl-3-oxoquinoxaline-2-carboxylate (1 g.) in propan-1-ol (50 c.c.) and the whole was refluxed in the dark for 72 hr. in an oxygen-free nitrogen atmosphere. The precipitate was collected and washed with ether. This material was shown by paper chromatography and light absorption to contain a small quantity of "lumiflavin 2-imine". This quantity of product was augmented by refluxing a yellow pigment, the main product of the reaction, in methanol.

3,4-Dihydro-6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline--2-carboxylic Acid.[87] - A solution of riboflavin (10 g.) in N-sodium hydroxide (300 c.c.) was heated for 1 hr. at 90°. The

solution was filtered, cooled, neutralised with acetic acid and left to stand for 3 days at 5°. The solid was collected and purified by dissolving in hot water, treating with charcoal, and adding an equal volume of alcohol to the filtrate. The resulting crystals (4.9 g.) of the sodium salt of the required carboxylic acid were collected and dried to constant weight at 95°, m.p. 243-244° (lit.[87], 242-243°).

The sodium salt (4.9 g.) was dissolved in hot water (150 c.c.) and treated with an excess of 10% sulphuric acid. After standing at 5° the yellow acid was collected and recrystallised from ethanol to give bright yellow crystals (2.55 g.), m.p. 115-120° (lit.[87], 183-183.5°).

Methyl 3,4-Dihydro-6,7-dimethyl-3-oxo-4-D-ribityl-quinoxaline -2-carboxylate. -

(a) To a solution of 3,4-dihydro-6,7-dimethyl-3-oxo--4-D-ribitylquinoxaline-2-carboxylic acid (4.6 g.) in the minimum quantity of methanol was added ethereal diazomethane (8 equivs.). After 30 min., the solution was evaporated to dryness *in vacuo* and the residue recrystallised from methanol, to give the methyl ester (4.25 g., 89%) as pale yellow needles, m.p. 185-186° (Found: C,55.9; H,6.3; N,7.6. $C_{17}H_{22}O_7N_2$ requires C,55.7; H,6.1; N,7.7%).

(b) 3,4-Dihydro-6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline--2-carboxylic acid (11 g.) was recrystallised from the minimum

quantity of methanol to give bright yellow needles (9 g.), m.p. 183°, identical in paper chromatography and ultra-violet and infrared absorption with the methyl ester prepared by the above reaction.

2,3,4,5,1',2',3',4'-Octahydro-2-imino-6',7'-dimethyl--3',5-dioxo-4'-D-ribitylglyoxaline-4-spiro-2'-quinoxaline. - To a solution from sodium (0.52 g.) in dry propan-1-ol (12.5 c.c.) was added dry powdered guanidine hydrochloride (0.225 g.) in propan-1-ol (12.5 c.c.). The mixture, after filtration, was added to a solution of methyl 3,4-dihydro-6,7-dimethyl-3-oxo--4-D-ribitylquinoxaline-2-carboxylate (0.25 g.) in dry propan-1-ol (12.5 c.c.) and kept at 25° for 20 hr. The cream-coloured precipitate was collected, washed with a little water, and dried, to give the spiro-compound (0.2 g., 75%), m.p. 183-186° (Found: C,48.3; H,6.6; N,16.9. $C_{17}H_{23}O_6N_5,1.5H_2O$ requires C,48.6; H,6.2; N,16.7%).

A solution of the spiro-compound in 2N-hydrochloric acid gave the hydrochloride, as yellow needles, m.p. 227-229° (from 2N-hydrochloric acid) (Found: C,47.7; H,5.9; N,16.7. $C_{17}H_{23}O_6N_5,HCl$ requires C,47.5; H,5.6; N,16.3%).

2-Amino-4-hydroxy-7,8-dimethylbenzo[g]pteridine. - Methyl 3,4-dihydro-6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline-2--carboxylate (1 g.) in dry propan-1-ol (50 c.c.) was treated with

guanidine (from 0.85 g. of the hydrochloride and 0.2 g. of sodium) in propan-1-ol (60 c.c.), and the mixture was refluxed for 90 hr. The resulting solution was evaporated to dryness in vacuo, and the residue was dissolved in water, and 2N-hydrochloric acid was added to give pH6. The yellow precipitate (0.66 g.) was collected, washed with water, dried, and recrystallised from dimethylformamide to give the benzopteridine as yellow needles, m.p. $> 350°$ (Found: C,59.7; H,4.2; N,28.6. Calc. for $C_{12}H_{11}ON_5$: C,59.7; H,4.6; N,29.0%).

2,10-Dihydro-4-hydroxy-2-imino-7,8-dimethyl-10-D--ribitylbenzo[g]pteridine. ("Riboflavin 2-imine"). - 3,4-Dihydro--6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline-2-carboxylate (1 g.) in dry n-propanol (50 c.c.) was condensed with guanidine (from 0.85 g. of hydrochloride and 0.2 g. of sodium by refluxing the mixture for 72 hr., under nitrogen, in the dark. The mixture was filtered while hot, and the crude orange-brown product (1.25 g.) was washed with ether and dried. This material, shown by paper chromatography to contain riboflavin 2-imine, was purified by one of the following methods.

(a) The crude material (1.25 g.) was dissolved in 50% acetic acid (300 c.c.) and the solution was neutralised with solid sodium hydrogen carbonate. The red insoluble material which separated was removed by centrifugation, and the supernatant solution was extracted with phenol (total 300 g.). Ether (1 l.)

was added to the phenolic extracts which were re-extracted with water (90 c.c.). This aqueous solution deposited overnight a red product shown by paper chromatography to be largely riboflavin 2-imine. This was dissolved in the minimum of 5% acetic acid and chromatographed on Florisil (50 g.) in the dark. The column was washed with 5% acetic acid (100 c.c.) and the product finally eluted with 1:9-pyridine-water (150 c.c.). The eluate was taken to dryness in vacuo (rotary evaporator), and the residue dissolved in the minimum quantity of 2N-hydrochloric acid. Addition of solid sodium hydrogen carbonate to pH 7 gave the isoalloxazine 2-imine ("riboflavin 2-imine") as an orange powder (0.15 g., 15%) (Found: C,54.1; H,5.7; N,18.2. $C_{17}H_{21}O_6N_5$ requires C,54.4; H,5.6; N,18.7%).

(b) The crude product (1.2 g.) was dissolved in 2N-hydrochloric acid (100 c.c.). To the filtered solution were added a few drops of hydrogen peroxide to bleach any soluble impurities, and 10% aqueous sodium hydrogen carbonate was added to give pH7. The resulting mixture was heated at 100° for 2 hr. to coagulate the precipitate, which was collected after being kept overnight at 0°. This procedure was repeated several times and finally gave riboflavin 2-imine (0.15 g.) as an orange powder. The infrared spectrum was identical with that of the specimen prepared as above.

Treatment of Isoalloxazines 2-imines with Alkali and Heat. -

(a) The imine (2 mg.) was heated in N-sodium hydroxide (1c.c.) at 100° for 1 hr. in the dark. The products were examined by paper chromatography and ultraviolet spectroscopy. Riboflavin 2-imine and lumiflavin 2-imine were converted completely into 2-aminolumichrome; and the 10-methylisoalloxazine 2-imine was converted into 2-aminoalloxazine.

(b) The treatment with alkali was repeated at room temperature in the dark. After three days riboflavin 2-imine was partially converted to 2-aminolumichrome and lumiflavin 2--imine, lumiflavin-2-imine was unchanged owing to its almost complete insolubility in N-sodium hydroxide, and the 10-methyl isoalloxazine 2-imine was entirely converted to 2-aminoalloxazine.

(c) Procedure (a) was repeated using water in place of N-sodium hydroxide and paper chromatography showed no change in any case.

Photolysis of Riboflavin 2-imine. - Riboflavin 2-imine in 0.1N-hydrochloric acid (5 c.c.) was irradiated for 16 hr. with ultraviolet light (Hanovia U.V. S.500). Paper chromatography and ultraviolet spectroscopy then showed the presence of 2-aminolumichrome and a little unchanged imine.

Similar photolysis in 0.5N-sodium hydroxide yielded

lumiflavin 2-imine, 2-aminolumichrome, and a product of unknown structure whose absorption spectrum (max. at 210, 270 and 370 mu at pH1), indicates that it is probably a quinoxaline.

Photolysis of Lumiflavin 2-imine. - Lumiflavin 2-imine (3 mg.) in 0.1N-hydrochloric acid (5 c.c.) was irradiated for 16 hr. with ultraviolet light. Paper chromatography and ultraviolet spectroscopy then showed the product of the reaction to be 2-aminolumichrome.

No photolysis of lumiflavin 2-imine in alkali was investigated owing to its insolubility.

Investigation of Blue-Fluorescing Compound. -

(a) In the above synthesis of riboflavin 2-imine an impurity with a blue fluorescence when viewed in ultraviolet light persisted. A sample (0.2 g.) of the crude reaction product was therefore chromatographed on a column of cellulose powder (Whatman No. 1; 50 g.) with butan-1-ol-water-acetic acid (63:27:10) as solvent. A blue-fluorescing band was eluted by this method and was shown to be pure by paper chromatography. The eluate of this band was therefore taken to dryness to give (LXXXII) as a buff-coloured solid (20 mg.) R_f value 0.59 in butan-1-ol-acetic acid-water (160:40:75) (Found: C,55.28; H,6.46. Calc. for $C_{15}H_{20}N_2O_6$. C,55.55, H,6.22%).

(b) The reaction mixture of the ribityl spiro compound was examined using paper chromatography and was shown to contain a blue-fluorescing spot, R_f value 0.59 in the above mentioned solvent system.

(c) 3,4-dihydro-6,7-dimethyl-3-oxo-4-D-ribityl quinoxaline-2-carboxylic acid (10 mg.) was dissolved in 0.1N-sodium hydroxide (5 c.c.) and irradiated in the usual manner for 16 hr.

The same procedure was repeated using 0.1N--hydrochloric acid in place of sodium hydroxide.

Both reactions on paper chromatography showed the presence of a blue fluorescing compound of R_f value 0.58 in the above solvent system.

(d) 3,4-Dihydro-6,7-dimethyl-3-oxo-4-D-ribitylquinoxaline--2-carboxylic acid (0.5 g.) was dissolved in dilute acetic acid and heated at 90°. Hydrogen peroxide was added dropwise until evolution of gas had subsided. The reaction mixture was then allowed to stand for 2 hr. at 90° during which time the yellow colour of the solution gradually weakened in intensity. On cooling a white microcrystalline solid was precipitated (0.32 g.) which showed an R_f value of 0.58 on paper chromatography using the above solvent system, m.p. 257-259° (lit.,[90] m.p.257-260°).

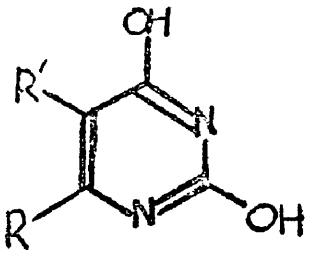
P A R T II

Chemical Studies of the Biosynthesis of Riboflavin.

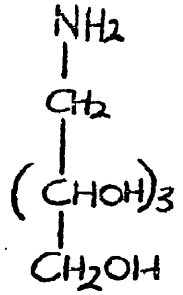
Theoretical

Synthesis of Pyrimidine Precursor.

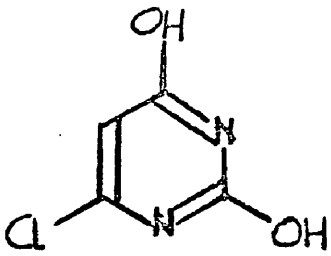
The implication of Goodwin's observation[48] that riboflavin 2-imine neither promotes nor inhibits flavinogenesis in *E. ashbyii* is that deamination in the route guanine ⟶ riboflavin must take place prior to formation of the condensed ring system of riboflavin. If one forgets for the moment the question of possible opening of the pyrimidine ring during the transformation and simplifies the sequence, as does Masuda,[52] to one of purine ⟶ 5-amino-4-substituted amino pyrimidine ⟶ pteridine ⟶ riboflavin, then it is obvious that the outstanding intermediate is the diaminopyrimidine. It seemed likely that this compound was 5-amino-2,6-dihydroxy-4-D-ribitylaminopyrimidine (LXXXIII, R = D-ribitylamino, R'=NH_2) and it was therefore decided to investigate the synthesis of this compound.



LXXXIII



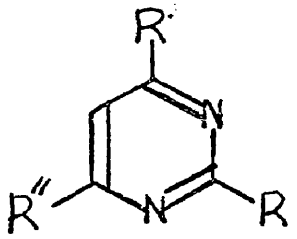
LXXXIV



LXXXV

The proposed synthesis involved condensation of D-ribitylamine (LXXXIV), a known sugar amine,[94,98,96] with 4-chloro-2,6-dihydroxy pyrimidine (LXXXV, "4-chlorouracil"). The pyrimidine product of this reaction, 2,6-dihydroxy-4-D-ribitylaminopyrimidine

(LXXXIII, R = D-ribitylamino, R' = H), would then be nitrosated to give the corresponding 5-nitrosopyrimidine (LXXXIII, R = D--ribitylamino, R' = NO) which on reduction either chemically or catalytically would give the desired 5-amino compound (LXXXIII, R = D-ribitylamino, R' = NH_2),

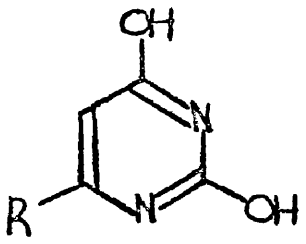


LXXXVI

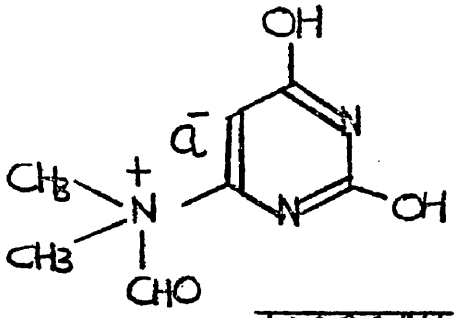
2,4,6-Trihydroxypyrimidine (LXXXVI, R=R'=R'' = OH, "barbituric acid") was converted[97] to its trichloro derivative (LXXXVI, R=R'=R'' = Cl). Alkaline hydrolysis of this trichloropyrimidine gave 4-chlorouracil (LXXXV). This one-step conversion was recommended by Langley[98] and is superior to the two-step literature method.

At this stage it was decided that a model synthesis using commercially available β-hydroxy ethylamine instead of D-ribityl amine would prove valuable. When 4-chlorouracil (LXXXV) and two equivalents of β-hydroxyethylamine were refluxed together using water as solvent the pyrimidine starting material was recovered. The reaction was repeated using N,N-dimethyl formamide and a white crystalline pyrimidine derivative was isolated in good yield. This product did not, however, analyse for the desired 4-β-hydroxyethylaminouracil (LXXXVII, R = β-hydroxyethylamino), but rather analysed for the corresponding 4-dimethylaminopyrimidine

(LXXXVII, R = dimethylamino). This dimethylamino compound was therefore prepared by an unambiguous method, namely a sealed tube condensation of 4-chlorouracil and dimethylamine at elevated temperature. The products from both condensations were identical in paper chromatography, ultraviolet and infrared absorption spectra, and in elementary analysis. This same compound, 4-dimethylamino-2,6-dihydroxypyrimidine, was detected when 4-chlorouracil was refluxed with N,N-dimethyl formamide in the absence of β-hydroxyethylamine.



LXXXVII

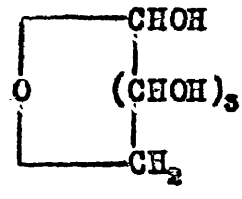


LXXXVIII

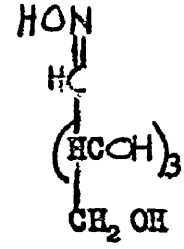
The mechanism for the condensation of N,N-dimethyl formamide and 4-chlorouracil is not obvious, but it may proceed <u>via</u> the quaternary salt (LXXXVIII).

Condensation of 4-chlorouracil and β-hydroxyethylamine was accomplished in an aqueous medium in a sealed-tube reaction at high temperature, and gave 2,6-dihydroxy-4-β-hydroxyethylamino-pyrimidine (LXXXVII, R = β-hydroxyethylamino), which on treatment with nitrous acid at low temperature gave the desired purple nitroso derivative (LXXXIII, R = β-hydroxyethylamino, R' = NO).

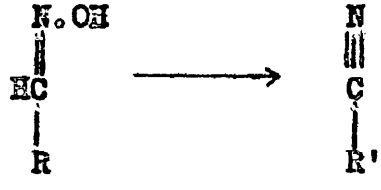
Two methods for the synthesis of D-ribitylamine are given in the literature but of these the more direct method of Folkers et al.[96,97] was rejected as the products were heavily discoloured. The method adopted was that of Kuhn, et al.[94,99] which consisted of converting D-ribose (LXXXIX) to its oxime[99] (XC) and then catalytically reducing the oxime to the desired amine.[94] This procedure was followed and the yield of amine was estimated by titration in the recommended manner,[94] but none of the known crystalline derivatives[94,100] of D-ribityl amine could be formed.



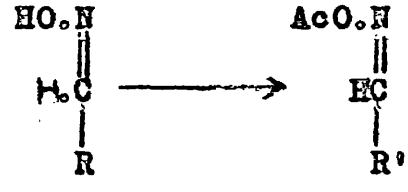
(LXXXIX)



(XC)



(XCI) (XCII)



(XCIII) (XCIV)

In an attempt to form the tetra-acetate of D-ribitylamine acetylation of D-ribose oxime, using acetic anhydride/pyridine, was investigated. The product, however, was a gum which proved intractible, probably explaining the surprising absence of any literature report of the acetylation of this important sugar oxime. It is known[101] that on acetylation anti oximes (XCI, R = sugar chain) tend to dehydrate to give the corresponding acetylated nitrile

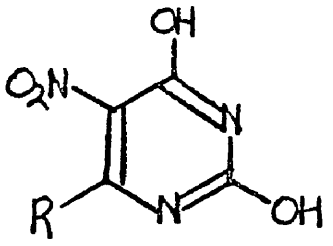
(XCII, R' = acetylated sugar chain), whereas with syn oximes (XCIII, R = sugar chain) there is the possibility of getting the fully acetylated oxime (XCIV, R' = acetylated sugar chain). The infrared spectrum of the above gum shows acetate and oxime bands, but no hydroxyl or nitrile bands thus indicating that D-ribose oxime is a syn oxime.

As reduction of D-ribose oxime was not giving the reported crystalline derivatives of D-ribityl amine the product of the reaction was further investigated by chromatography on a cation exchange resin, the product being eluted with oxalic acid. The amine containing fractions from the column were detected using the ninhydrin test,[102] and these fractions were finally examined using paper chromatography. The papers were developed by spraying with sodium periodate solution and, after removal of iodine, detecting any aldehyde produced by means of Schiff's reagent. These techniques showed that there were at least two products in the reaction mixture.

This being so it was decided that the crude reaction product from reduction of D-ribose oxime, after removal of solvent at low temperature, should be reacted with 4-chlorouracil and that any pyrimidine mixture should then be separated.

Reaction of the crude D-ribitylamine gum and 4-chlorouracil at high temperature in a sealed tube gave, however, a charred

solution which on paper chromatography appeared void of any pyrimidine product. For this reason this approach was abandoned. (It is only fair to add that Plaut and Maley[56] subsequently achieved this condensation under slightly less drastic conditions, but here again the reaction mixture was not promising and required ion-exchange chromatography before yielding the required pyrimidine derivative [LXXXVII, R = D-ribitylamino]).



XCV

Nitration of 4-chlorouracil using fuming nitric acid with concentrated sulphuric acid readily gave 4-chloro-2,6-dihydroxy-5-nitropyrimidine (XCV, R = Cl), a very reactive 4-chloropyrimidine. Treatment of (XCV, R = Cl) with water, ammonia, β-hydroxyethylamine, and methylamine readily gave the corresponding 4-hydroxy (XCV, R = OH), 4-amino (XCV, R = NH_2), 4-β-hydroxyethylamino (XCV, R = $NH.CH_2CH_2OH$), and 4-methylamino (XCV, R = $NHCH_3$) pyrimidines. Condensation of (XCV, R = Cl) with crude D-ribitylamine, hoever, gave a mixture of products from which a small amount of the 4-aminopyrimidine (XCV, R = NH_2) was readily separated. The remainder of the reaction mixture, which showed two product spots on paper chromatography, was absorbed on an anion exchange resin and the products eluted using ammonium formate buffer

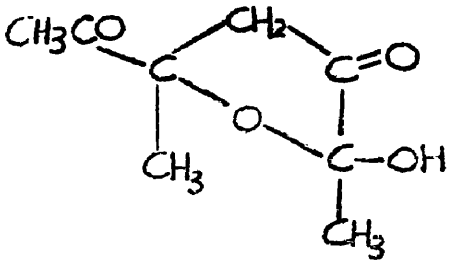
at various pH values. Buffer at pH7 eluted a nitro-pyrimidine product which is as yet unidentified. Buffer at pH4 eluted a second nitro-pyrimidine product in large yield which on isolation proved to be the required 2,6-dihydroxy-5-nitro-4-D-ribitylamino-pyrimidine (XCV, R = D-ribitylamino).

It was thought that the unidentified pyrimidine product of the condensation might be the corresponding 4-di-D-ribitylamino-pyrimidine (XCV, R = di-D-ribitylamino) and to test this theory 4-chloro-5-nitrouracil (XCV, R = Cl) was condensed with dimethylamine. The resulting 4-dimethylamino-2,6-dihydroxy-5-nitro-pyrimidine (XCV, R = dimethylamino)showed, however, an ultraviolet absorption spectrum which was markedly different (see Table 2) from that of the unidentified nitro-pyrimidine, whose absorption spectrum was more akin to that of a 4-monoalkylamino-2,6-dihydroxy--5-nitro-pyrimidine. It is therefore unlikely that this compound is a 4-dialkylaminopyrimidine.

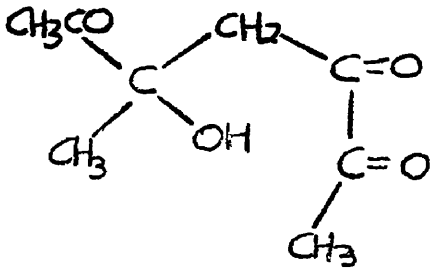
Synthesis of Aromatic Precursors.

The dimerisation of diacetyl which is reported in the literature[81,103] was repeated but no crystals of the otherwise satisfactory product could be obtained. Attempts to self-condense diacetyl using an anion exchange resin in its hydroxyl form produced a crystalline aldol which was different in its infrared spectrum from the dimer described by Birch.[81] The dimer was assigned[81]

structure (XCVI) largely on the basis of its infrared spectrum. The latter showed peaks at 1764 cm.$^{-1}$ (assigned to a carbonyl group in a 5-membered ring), 1720 cm.$^{-1}$ (acetate carbonyl), and one at 3600 cm.$^{-1}$ (hydroxyl). It is therefore a semi-ketal derived from the open-chain hexane-trione (XCVII).



XCVI



XCVII

The product from the new condensation proved to be identical with a crystalline product which was obtained from an old bottle of diacetyl and it can therefore apparently be formed under very mild conditions. This new aldol was shown to be a trimer of diacetyl by elementary analysis and by molecular weight determination using the elevation of the boiling point technique. This result was supported by X-ray crystallographic studies which showed that the crystal cell was monoclinic and contained 12 diacetyl units. Since in a monoclinic cell the number of molecules is unlikely to be other than 2, 4, or 8, this indicates a trimer (The author is indebted to Dr. Speakman of the University of Glasgow for carrying out this preliminary X-ray study). Final evidence for the product

being a trimer came from its nuclear magnetic resonance spectrum which showed a total number of hydrogen containing groups consistent only with three diacetyl molecules. Thus the N.M.R. data[E] showed that the trimer contains two different acetyl groups (7.741, 7.804 p.p.m.) three methyl groups (8.608, 8.682, 8.708 all of a type β to oxygen, only one CH_2 group (doublets at 6.87 and 8.24, AB type, J_{AB} = 13.5 c s), and only a single OH group of such a low value 5.52τ at 6.0% w/v in carbon tetrachloride that is necessary to invoke rather strong internal hydrogen--bonding. (The author is indebted to Dr. G. V. D. Tiers of the Minnesota Mining and Manufacturing Company for the above N.M.R. data). This evidence agrees well with the infrared data which showed one type of carbonyl at 1706 $cm.^{-1}$ typical of an open-chain ketone, an intramolecularly H-bonded hydroxyl band at 3436 $cm.^{-1}$ and a C-methyl band at 2976 $cm.^{-1}$

Since in the trimer there is only one CH_2 group and one OH group then it follows that only two diacetyl molecules can be linked by aldol type linkage. The hexane trione must therefore be a fundamental unit in the trimer molecule, and it is likely that the third molecule of diacetyl is linked by acetal linkages. Chart I shows various ways in which this can be done leading to four possible structures for the trimer (XCVIII→CI).

[E] Band positions are given as τ-values as defined by Tiers (J. Phys. Chem., 1958, 62, 1151).

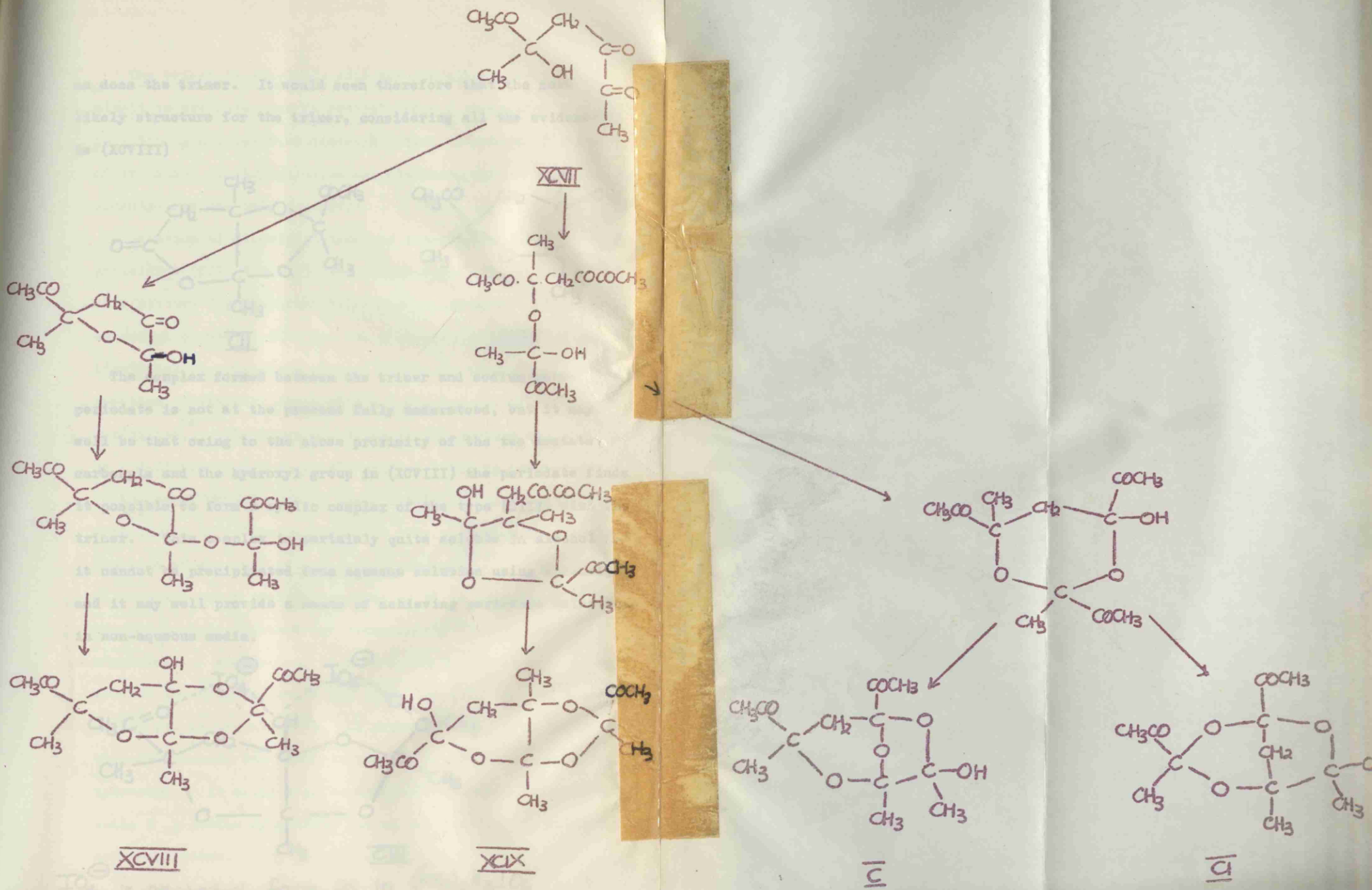
Examination of accurate molecular models ("Buchi" models) show that in structures (C) and (CI) the distance between hydroxyl and either carbonyl group is too great to permit the strong hydrogen bonding, which is indicated by both infrared and N.M.R. spectra. The distances, as measured on the model, range from 3.8Å. → 4.4Å., whereas Pauling[104] quotes an O——H .....O distance of 2.5-2.9Å. Similar measurements on models of structures (XCVIII) and (XCIX) give O——H .....O distances of the order given by Pauling for hydrogen-bonding.

Of these two possible structures (XCVIII) and (XCIX) the latter seems unlikely as the trimer does not undergo a normal type of periodate oxidation.

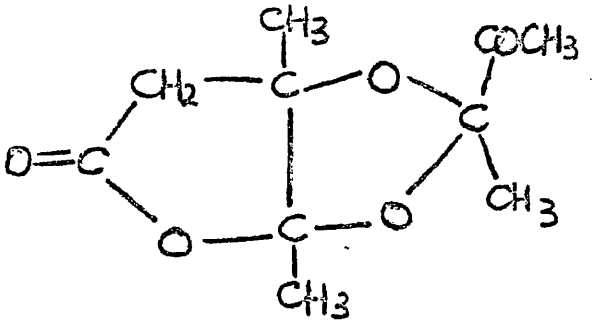
On treatment with sodium meta-periodate the trimer consumes exactly 2 moles as determined by titration with sodium arsenite, but this is not a normal oxidation as the periodate is still available for oxidation of a normal cis-glycol such as ethylene glycol. Further evidence that the trimer portion is unaffected in this pseudo-oxidation is the fact that the trimer still reacts, after the oxidation, with 4,5-diaminopyrimidines to give the same mixtures as it normally does (see later section).

If structure (XCIX) were the structure of the trimer it should consume 1 mole of periodate to give the cyclic ketone (CII). This does not happen since the cyclic ketone (CII) could not react with a 4,5-diaminopyrimidine in the same manner

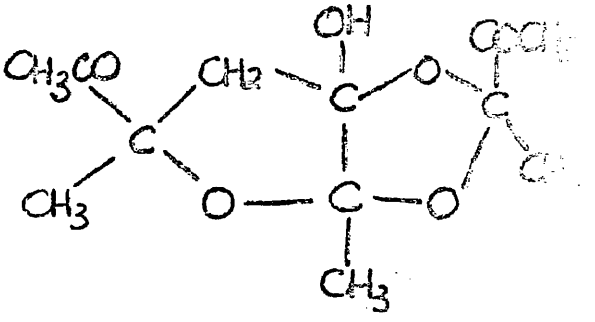
Chart I



as does the trimer. It would seem therefore that the most likely structure for the trimer, considering all the evidence, is (XCVIII)

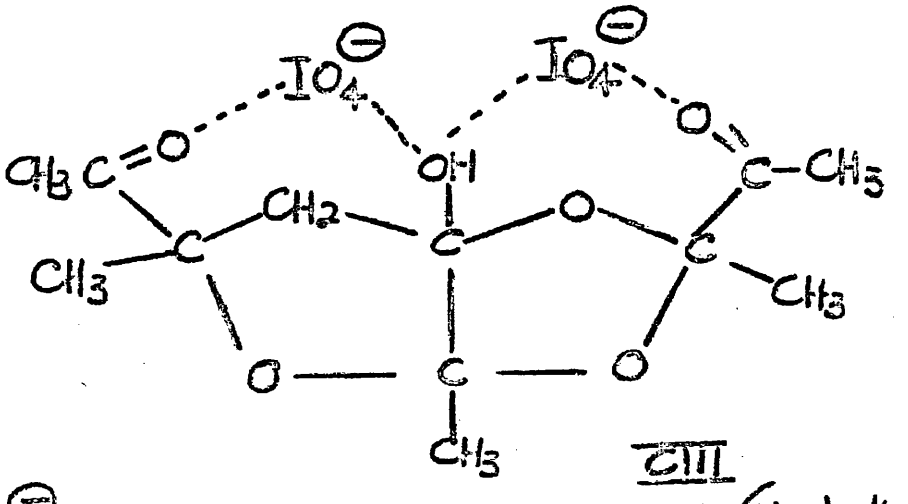


CII



XCVIII

The complex formed between the trimer and sodium meta-periodate is not at the present fully understood, but it may well be that owing to the close proximity of the two acetate carbonyls and the hydroxyl group in (XCVIII) the periodate finds it possible to form a cyclic complex of the type (CIII) with the trimer. This complex is certainly quite soluble in alcohol for it cannot be precipitated from aqueous solution using ethanol, and it may well provide a means of achieving periodate oxidations in non-aqueous media.

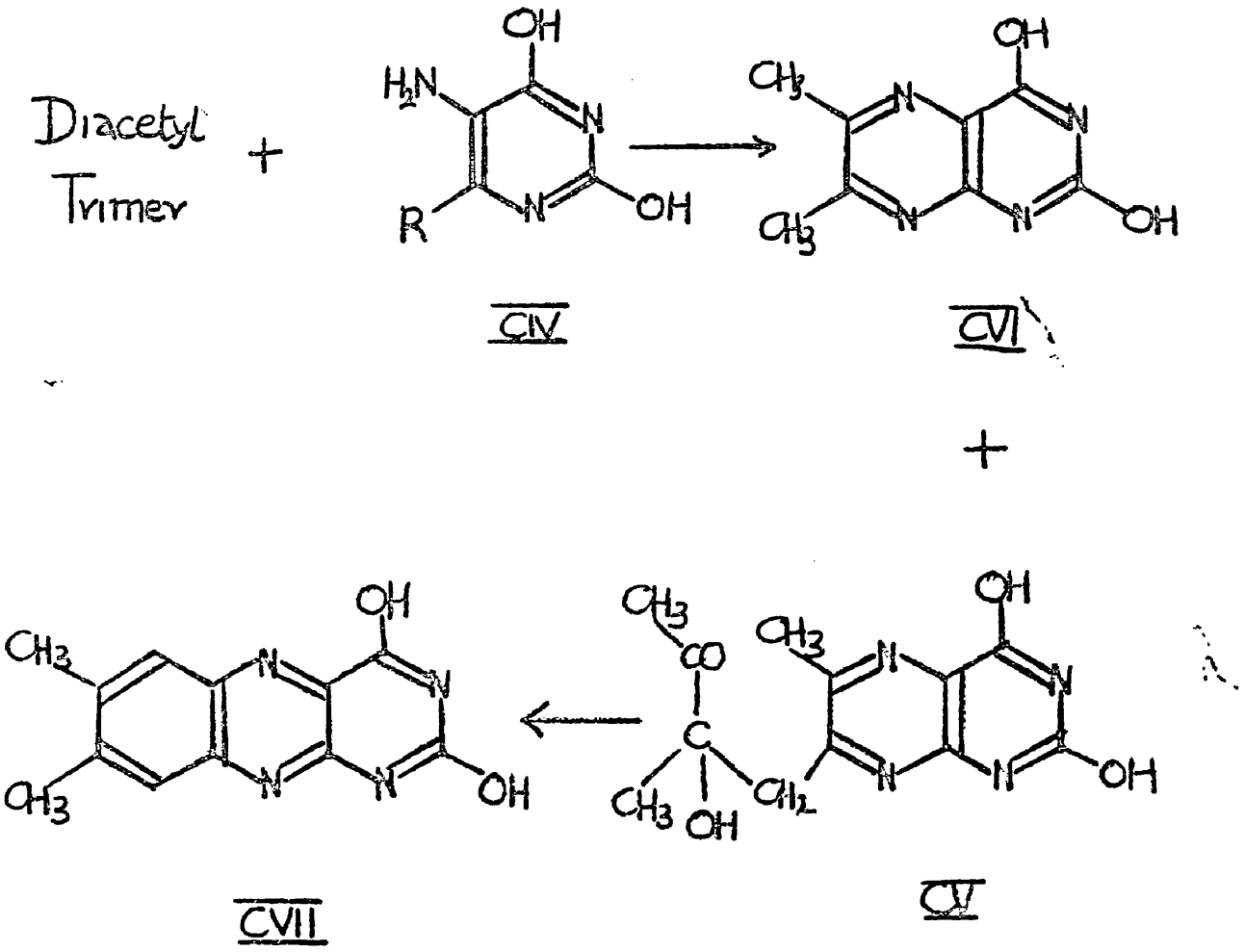


CIII

$IO_4^{\ominus}$ in octahedral form as in $(NH_4)_2H_3IO_6$

The trimer is stable in acid but readily degrades in alkali in the cold, and in general shows a pronounced tendancy to split up and react as diacetyl. Thus on attempted formation of an oxime, dimethylglyoxime was obtained, and on acetylation diacetyl was the only product.

Reaction of diacetyl trimer and 4,5-diamino-2,6-dihydroxy-pyrimidine (CIV, R = NH_2) gave a mixture of pteridines. From this mixture 2,4-dihydroxy-7-(2'-hydroxy-2'-methyl-3'-oxobutyl)--6-methyl pteridine (CV) (or the 6 isomer) was isolated and identified by comparison with an authentic sample prepared by reaction of diacetyl dimer with 4,5-diamino-2,6-dihydroxy-pyrimidine.[51] The other pteridine in the mixture was shown to be 2,4-dihydroxy-6,7-dimethylpteridine (CVI) by comparison (using paper chromatography and ultraviolet absorption spectroscopy) with an authentic sample of this pteridine obtained from reaction of diacetyl with 4,5-diaminouracil.[51,103] The trimer of diacetyl was therefore reacting as if it were dimer and monomer loosely combined and consequently free to react separately. At the same time as these reactions were being studied the cyclisation of the pteridine (CV) to lumichrome (CVII) was re-investigated. As reported[51] by Birch and Moye this cyclisation was found to proceed readily using N-sodium hydroxide. It could also be achieved, although less readily, using N_{10} sodium hydroxide, but no cyclisation was achieved in acid conditions.

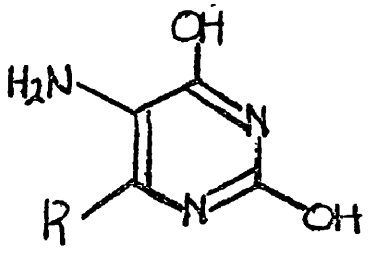


Condensation of Pyrimidine and Aromatic Precursors.

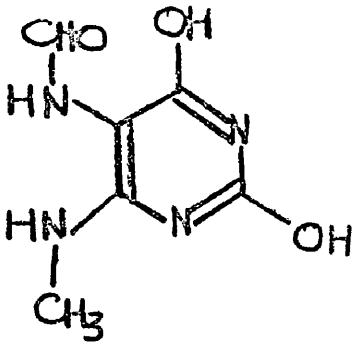
The nitro group in the 5-nitro-4-substituted aminopyrimidines (XCV) was readily reduced catalytically, but as soon as air was allowed into the hydrogenation flask the resulting 5-amino-4--substituted aminouracil underwent self-condensation. For this reason isolation of any of these diaminopyrimidines proved impossible, and in most cases a chemical reducing agent such as sodium dithionite in slight excess, or zinc dust in dilute mineral acid, was used in preference to a catalytic method of reduction. The structure of the self-condensation product is discussed later in this thesis.

In an attempt to synthesise a stable derivative of the 5-amino-4-substituted aminouracils (CIV, R = NHX) 2,6-dihydroxy--4-β-hydroxyethylamino-5-nitropyrimidine (XCV, R = $NHCH_2CH_2OH$) was reduced using zinc dust and concentrated formic acid. After removal of the zinc residues from the reaction mixture a white ether-insoluble pyrimidine was obtained. This pyrimidine was readily recrystallised from water as white needles which analysed in agreement with a di-formyl derivative of 5-amino--2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine. The crystalline product appeared to be quite stable in the solid state, but in hot aqueous solution the self-condensation reaction which is typical of 5-amino-4-substituted aminouracils (see below) was observed. When the above treatment with zinc dust and formic acid was repeated using 2,6-dihydroxy-4-methylamino-5-nitropyrimidine (XCV, R = $NHCH_3$) a white ether-insoluble product was once again obtained. This product also gave white needles on recrystallisation from water but on this occasion the needles analysed for the mono-formyl derivative of 5-amino-2,6-dihydroxy--4-methyl aminopyrimidine. The formyl product was on this occasion stable both in the solid state and in hot aqueous solution at pH7. It therefore seems likely that the mono-formyl derivative of 5-amino-2,6-dihydroxy-4-methylaminopyrimidine is (CVIII), and that the di-formyl derivative of the 4-β-hydroxyethylamino analogue is (CIX), the extra formylation having taken place on the side-chain hydroxyl. This might well explain the pronounced

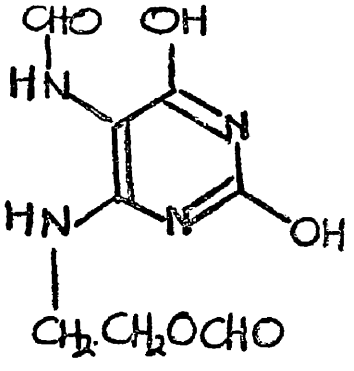
instability of the di-formyl derivative of the 4-β-hydroxyethyl-amino compound in that the formyl attached to the side chain alcohol may well be removed in hot aqueous solution at pH7 so liberating formic acid which lowers the pH of the solution and thus hydrolyses off the second formyl to give the unstable 5-amino-4-β-hydroxylethylaminouracil (CIV, R = $NHCH_2CH_2OH$). It was indeed observed that a hot aqueous solution of the di-formyl derivative of (CIV, R = $NHCH_2CH_2OH$) does show a pronounced drift towards an acid pH value.



CIV



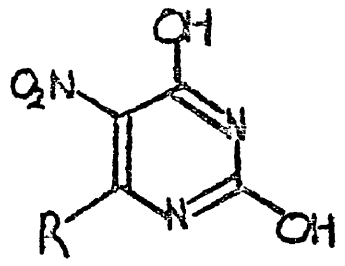
CVIII



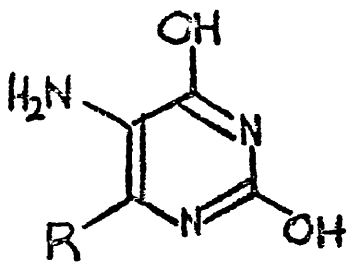
CIX

(a) Model experiments using the β-hydroxyethylaminopyrimidine (CIV, R = β-hydroxyethylamino).

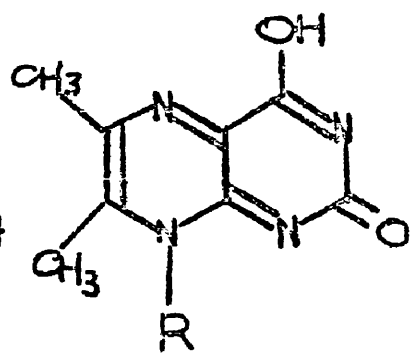
Hydrogenation of 2,6-dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (XCV, R = $NH.CH_2CH_2OH$) or its corresponding 5-nitroso analogue using platinum or palladium catalyst readily gave the 5-aminopyrimidine (CIV, R = $NH.CH_2.CH_2OH$) which was condensed with diacetyl in the absence of oxygen. From the reaction mixture the very soluble 2,8-dihydro-4-hydroxy-8-β--hydroxyethyl-6,7-dimethyl-2-oxopteridine (CX, R = CH_2CH_2OH) was isolated and characterised. This pteridine, whose U.V. absorption spectra were identical with those of Masuda's G-compound,[88] was also obtained as the sole product when diacetyl trimer was reacted with the 5-aminopyrimidine (CIV, R = $NHCH_2CH_2OH$) in an oxygen-free atmosphere in the presence of the platinum catalyst. It appears that diacetyl trimer breaks down under these conditions, and catalytic reduction of the nitro group was abandoned at this stage in favour of chemical methods.



XCV



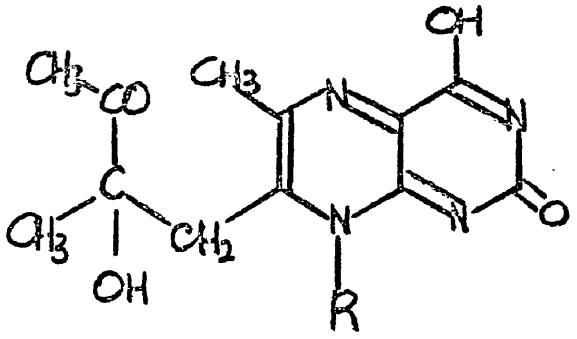
CIV



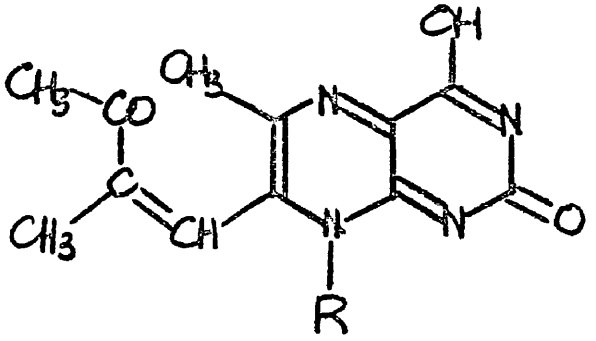
CX

The reaction of diacetyl dimer with 5-amino-2,6-dihydroxy--4-β-hydroxyethylaminopyrimidine (CIV, R = $NHCH_2CH_2OH$) was next studied at some length. When these compounds were reacted at pH4 and 90° for a quarter of an hour and the reaction mixture examined using paper chromatography it was found that there were two products of the reaction. The less mobile product in solvent (A) was identical in fluorescence and in ultraviolet absorption spectra with the pteridine (CX, R = CH_2CH_2OH), but was quite distinct from it in R_F value. For these reasons this new pteridine was assumed to be the 7-(2'-hydroxy-2'-methyl-3'-oxobutyl) pteridine (CXI, R = $CH_2.CH_2OH$) (or the 6-isomer). This pteridine has not been isolated in crystalline form since the solid obtained from pure concentrated solutions proved very hygroscopic and surprisingly prone to decomposition. The more mobile product in solvent (A) was always present in very small amounts and exhibited an orange fluorescence when viewed in ultra-violet light. It was obtained in low yields as a readily crystalline orange solid by elution from large-scale paper chromatograms of the reaction mixture. Analysis of this orange-fluorescing compound indicated a molecular formula of $C_{14}H_{16}N_4O_4$. It could thus be either compound (CXII, R = CH_2CH_2OH) or (CXIII, R = CH_2CH_2OH). It is of interest that this orange-fluorescing compound cannot be converted to the 9-hydroxyethylisoalloxazine (CXIV, R = CH_2CH_2OH) by any of the procedures reported immediately

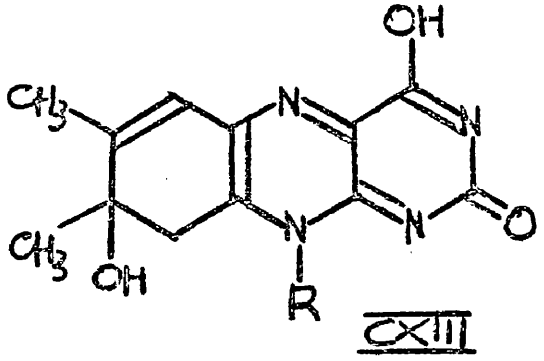
below for the conversion of the pteridine (CXI, R = CH_2CH_2OH) to this isoalloxazine, and it would seem that the structure (CXII, R = CH_2CH_2OH) is more in keeping with this observation than is structure (CXIII, R = CH_2CH_2OH).



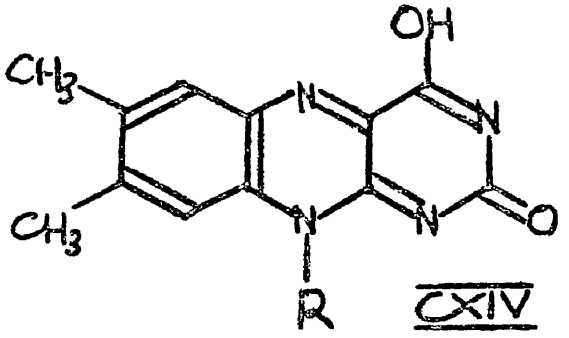
CXI



CXII



CXIII

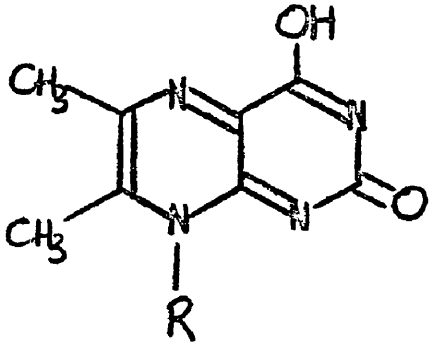


CXIV

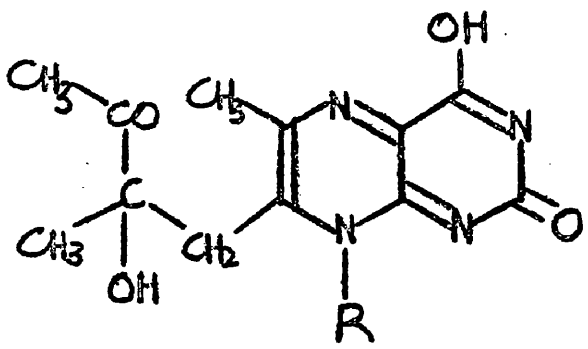
The reaction mixtures obtained above by condensation of diacetyl dimer and 5-amino-2,6-dihydroxy-4-β-hydroxyethylamino-pyrimidine were treated with either dilute alkali or acid of varying strengths. The main product of all such reactions was a very pure form of 6,7-dimethyl-9-β-hydroxyethylisoalloxazine (CXIV, R = CH_2CH_2OH). The ease with which this cyclisation takes place is quite astonishing and examination of the mother liquors of all these reactions showed that it was the yellow-fluorescing band corresponding to the pteridine (CXI, R = CH_2CH_2OH) which had

disappeared in the course of the cyclisation. In every case thorough comparison using infrared and ultraviolet spectroscopy, paper chromatography, and melting-point data was made of the product of the reaction and an authentic sample of 6,7-dimethyl-9-β-hydroxyethylisoalloxazine.

Attention was next focussed on the reaction of diacetyl trimer with 5-amino-2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine. The two compounds as in the previous case were reacted together at pH4 and 90° for 15 minutes, and the reaction mixture was examined using paper chromatography in the usual manner. This time the chromatogram exhibited three product bands. Two of these bands were identical with the two bands obtained in the dimer reaction, and the third product was identical in fluorescence ultraviolet absorption spectra, and R_F value with 2,8-dihydro-4--hydroxy-8-β-hydroxyethyl-6,7-dimethyl-2-oxopteridine (CX, R = $CH_2 . CH_2 OH$). As in the case of the reaction of the trimer and 4,5-diaminouracil the trimer is reacting as if it were a mixture of dimer and monomer.



CX

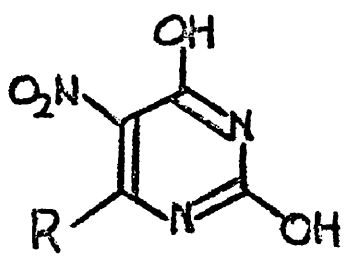


CXI

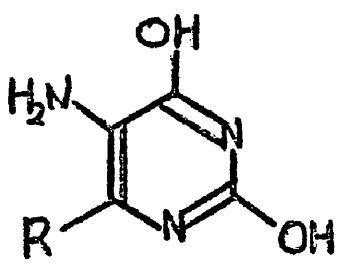
As before various trimer reaction mixtures were treated with either dilute alkali or different strengths of acid and heated for periods ranging from 30 min. to 8 hr. In all conditions formation of 6,7-dimethyl-9-β-hydroxyethylisoalloxazine took place although in the case of the dilute alkali cyclisation its presence was only demonstrated using paper chromatography and ultraviolet absorption spectroscopy. In the reactions conducted in acid solution the purity of the crystalline 9-hydroxyethylisoalloxazine which separated from the reaction mixture was if anything better than that of the same product obtained in the same manner in the dimer series. Examination of the mother liquors showed that in the cyclisation the pteridine (CXI, R = CH_2CH_2OH) tends to disappear, whereas the pteridine (CX, R = CH_2CH_2OH) remains unchanged in the reaction mixture.

(b) Model experiments using the methylaminopyrimidine (CIV, R = methylamino).

A series of reactions similar to that above involving the 4-β-hydroxyethylaminopyrimidine (CIV, R = $NH.CH_2CH_2OH$) was carried out using the 4-methylamino analogue (CIV, R = $NHCH_3$).



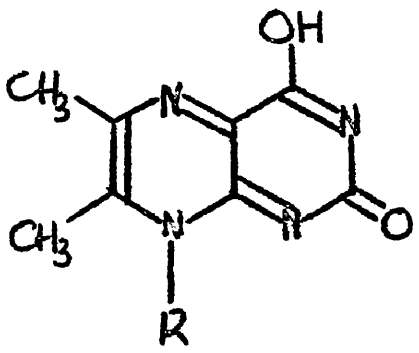
XCV



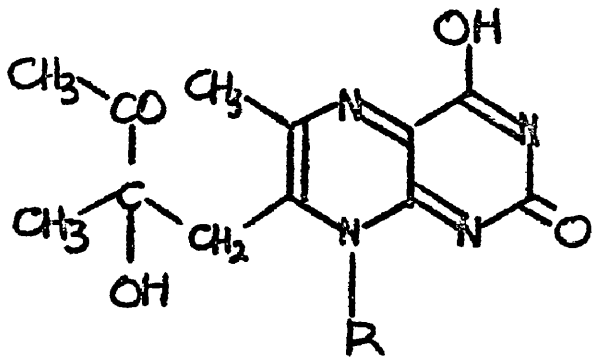
CIV

The reaction of diacetyl dimer and 5-amino-2,6-dihydroxy-4--methylaminopyrimidine (CIV, R = $NHCH_3$) was investigated and the reaction shown to give products analogous to those obtained in the reaction involving the pyrimidine (CIV, R = $NHCH_2CH_2OH$). Thus the known pteridine (CXI, R = CH_3) (or its 6-isomer)[61] and an orange-fluorescing compound which analysed for either (CXII, R = CH_3) or (CXIII, R = CH_3) were the products of the reaction. The pteridine (CXI, R = CH_3) was not isolated but was directly cyclised to lumiflavin (CXIV, R = CH_3) using acid or alkali. Once again the orange-fluorescing compound, which was always present in small amounts, did not undergo this cyclisation.

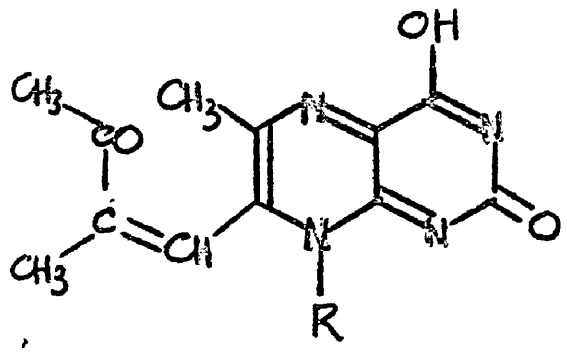
The above experiments are essentially a confirmation of the results of Birch and Moye,[61] but the conditions used for cyclisation of the pteridine intermediate are superior to those quoted in the literature.



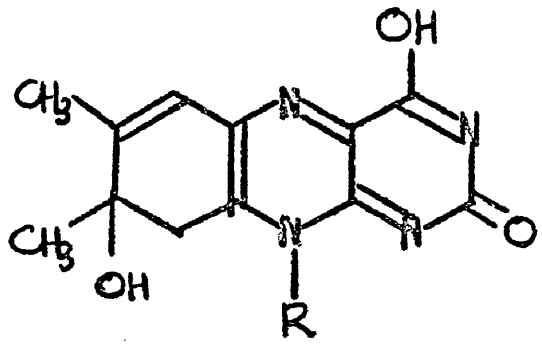
CX



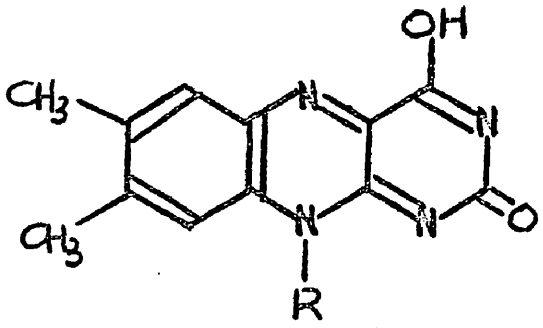
CXI



CXIII



CXIII



CXIV

The reaction of diacetyl trimer with the 5-aminopyrimidine (CIV, R = $NHCH_3$) followed the same course as the dimer reaction except that the reaction mixture contained 2,8-dihydro-4-hydroxy--2-oxo-6,7,8-trimethylpteridine (CX, R = CH_3) a known pteridine.[54] That this pteridine was the additional compound was checked by comparison of the reaction mixture with the condensation product from the reaction of diacetyl and the pyrimidine (CIV, R = CH_3). Treatment of the crude trimer reaction mixture with hydrochloric acid brought about cyclisation of the pteridine intermediate (CXI, R = CH_3) to lumiflavin.

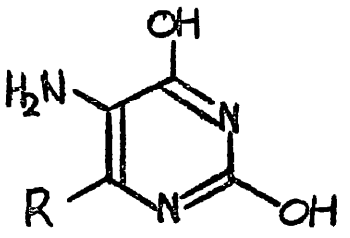
(c) Synthesis of riboflavin from pyrimidine and aromatic precursors.

This section deals with the reaction series involving the most important of the 5-amino-4-substituted aminouracils, the one which is probably a biological precursor of riboflavin, namely 5-amino-2,6-dihydroxy-4-D-ribitylaminopyrimidine (CIV, R = D--ribitylamino). The reaction of this pyrimidine with diacetyl dimer was therefore investigated.

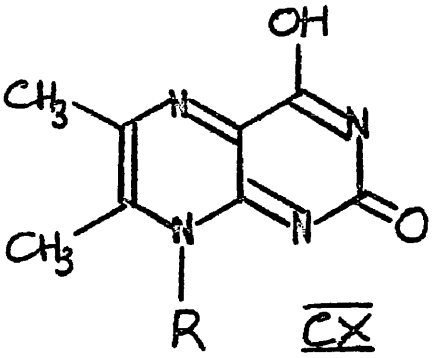
The reaction gave the expected mixture of two products. One of them, a yellow fluorescing compound, had an ultraviolet absorption spectrum similar to that of Kasuda's "G-compound" (CX, R = D-ribityl),[68] but differing from G-compound in R_F value in solvent system (A). The second product was an orange-fluorescent compound analogous to those obtained in the model experiments. Cyclisation of the yellow-fluorescing compound, which by analogy with the above model experiments is taken to be the pteridine (CXI, R = D-ribityl), to riboflavin (CXIV, R = D-ribityl) was readily achieved by treatment with either acid or alkali.

The corresponding reaction using diacetyl trimer differed in the usual way in that the reaction mixture contained an extra spot when examined on paper chromatography. This corresponded to the pteridine (CX, R = D-ribityl, "G-compound") which was synthesised unambiguously by condensation of diacetyl with the pyrimidine (CIV, R = D-ribitylamino). Riboflavin (CXIV, R = D-ribityl) was once

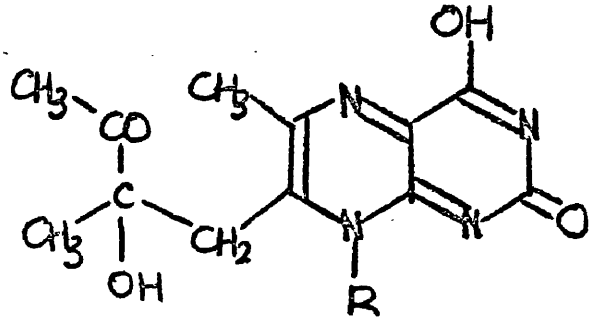
mors obtained from pteridine (CXI, R = D-ribityl) by cyclisation using acid or alkali.



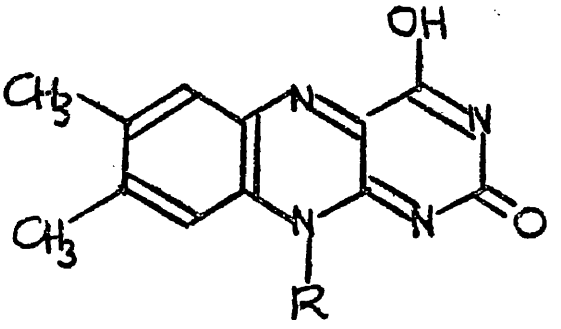
CIV



CX



CXI



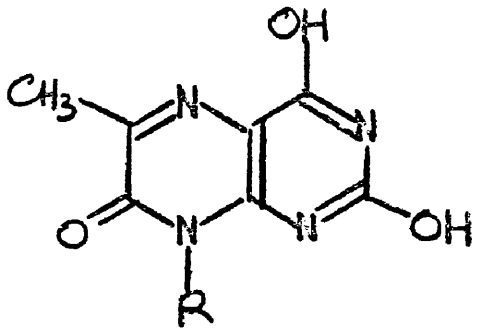
CXIV

(d) Syntheses of pteridines analogous to Masuda's "V-compound".

Condensation of 5-amino-4-β-hydroxyethylaminouracil (CIV, R = $NHCH_2CH_2OH$) with pyruvic acid gave 7,8-dihydro-2,4-dihydroxy--8-β-hydroxyethyl-6-methyl-7-oxopteridine (CXV, R = CH_2CH_2OH). Similar condensation of the 4-methylaminopyrimidine (CIV, R = $NHCH_3$) gave the 7-oxopteridine (CXV, R = CH_3). Both these pteridines showed ultraviolet absorption spectra identical with those given by Masuda[64] for his "V-compound" (CXV, R = D-ribityl).

At this point Maley and Plaut[65] reported the synthesis of "V-compound" itself by condensation of 5-amino-4-D-ribitylamino

uracil (CIV, R = D-ribityl) and pyruvic acid. Further work on the problem was therefore abandoned.

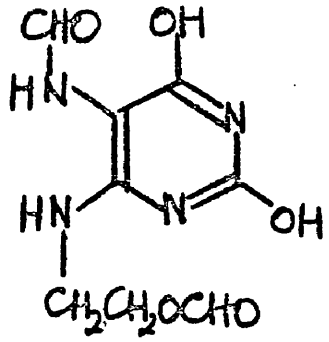


CXV

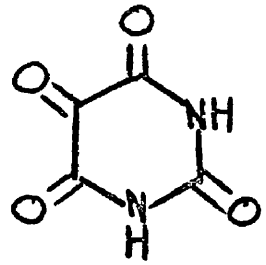
(e) Self-condensation products from 5-amino-4-substituted aminouracils (CIV, R = substituted amino).

The ease with which 5-amino-4-substituted aminouracils undergo self-condensation has already been mentioned. Thus the di-formyl derivative of 5-amino-2,6-dihydroxy-4-β-hydroxyethyl-aminopyrimidine (CIX) was heated for several hours in acid solution. A yellow crystalline product was obtained from this self-condensation reaction. This product had an analysis which indicated a pyrimidopteridine structure analogous to the compounds recently prepared by Taylor.[106] The ultraviolet spectrum of this self-condensation product was also in fair agreement with that of the compounds prepared by Taylor,[106] which were all derivatives of 2-aminopyrimidine, and were prepared by reaction of alloxan (CXVI) with pyrimidines of the type (CXVII, R = sugar amino or alkylamino). In earlier papers[107,108]

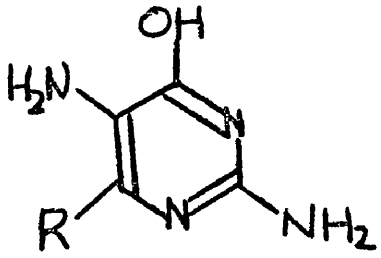
using 4,5-diaminopyrimidines and alloxan Taylor et al. had shown that structure (CXVIII) was preferred to (CXIX) for these pyrimidopteridines.



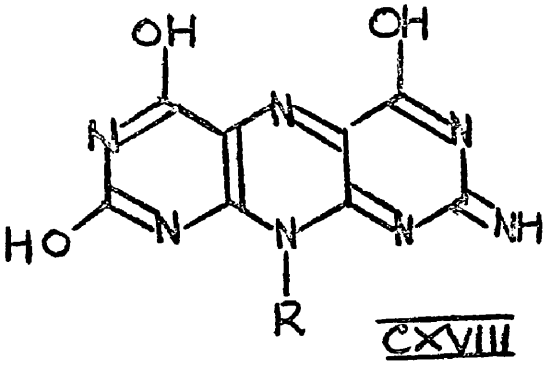
CIX



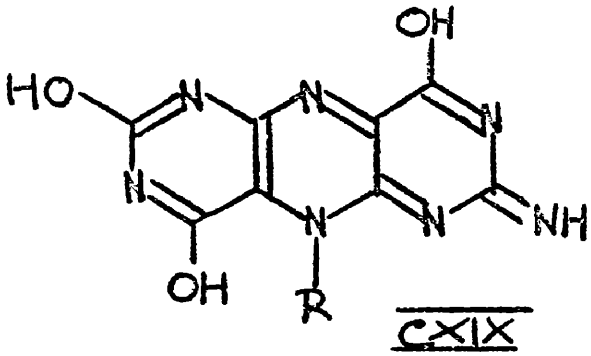
CXVI



CXVII



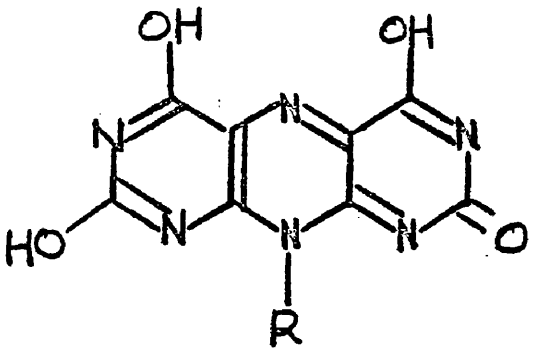
CXVIII



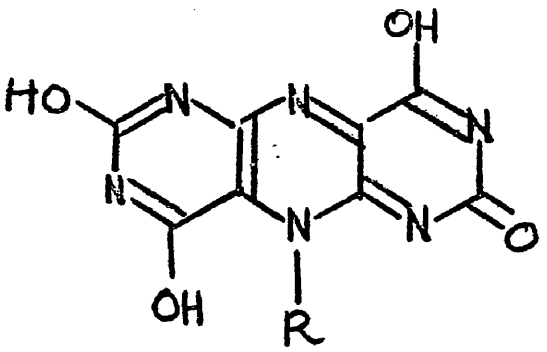
CXIX

Condensation of 5-amino-2,6-dihydroxy-4-β-hydroxyethylamino-pyrimidine with alloxan using the method of Taylor,[106] or a modification in which the 5-aminopyrimidine was made by reduction of the 5-nitro analogue with sodium dithionite rather than with zinc dust in dilute mineral acid, gave a pyrimido-pteridine for which by analogy with Taylor's work[106,107,108] the structure (CXX, R = CH_2CH_2OH) is preferred to the structure (CXXI, R = CH_2CH_2OH). This synthetic product was identical with the product obtained from the self-condensation reaction in

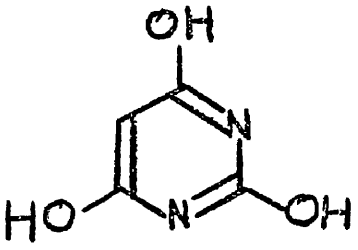
fluorescence, ultraviolet and infrared spectroscopy, and in R_F value on paper chromatography using solvent system (B). The self-condensation reaction therefore involves the elimination of 1 mole of ammonia and 1 mole of ethanolamine per two moles of pyrimidine.



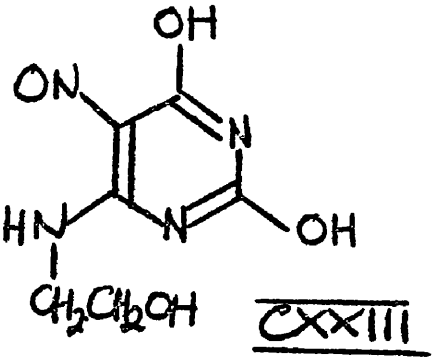
CXX



CXXI



CXXII



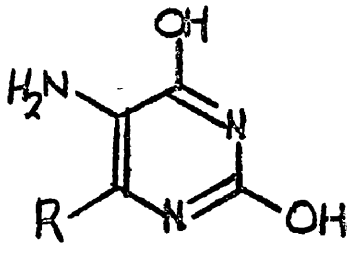
CXXIII

An unambiguous synthesis of the pyrimidopteridine (CXX, R = CH_2CH_2OH) using barbituric acid (CXXII) and 2,6-dihydroxy-4--β-hydroxyethylamino-5-nitrosopyrimidine (CXXIII) was attempted but the product proved to be a mixture of two compounds of very similar solubilities. Separation was achieved using paper chromatography in solvent system (B) and by repeated recrystallisation from dilute hydrochloric acid, and the less mobile

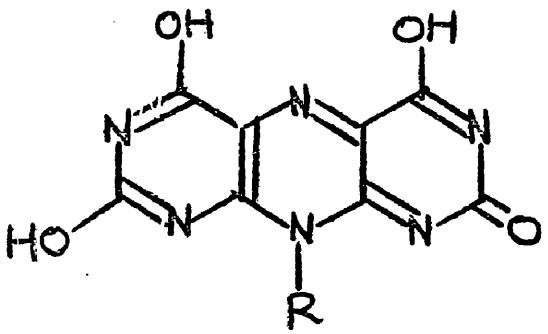
component of the mixture was shown to be identical with the self-condensation product in fluorescence, R_F value, and in its ultraviolet and infrared absorption spectra. It seems likely considering this evidence that the preferred structure (CXX, R = CH_2CH_2OH) is the correct one. Further evidence could be gained from degradative studies of these pyrimidopteridines.

In a similar way prolonged self-condensation of the 4-methylaminopyrimidine (CIV, R = $NHCH_3$) gave a pyrimidopteridine (CXX, R = CH_3) identical with that obtained by condensation of alloxan with the same pyrimidine (CIV, R = $NHCH_3$).

Reactions involving the 4-D-ribitylaminopyrimidine (CIV, R = D-ribitylamino) also gave a crystalline product which was readily identified as a pyrimidopteridine by its characteristic fluorescence. This pyrimidopteridine from self-condensation was identical, as in all other cases, with the product of the reaction of alloxan and the pyrimidine (CIV, R = D-ribityl-amino), and therefore has the structure (CXX, R = D-ribityl).



CIV



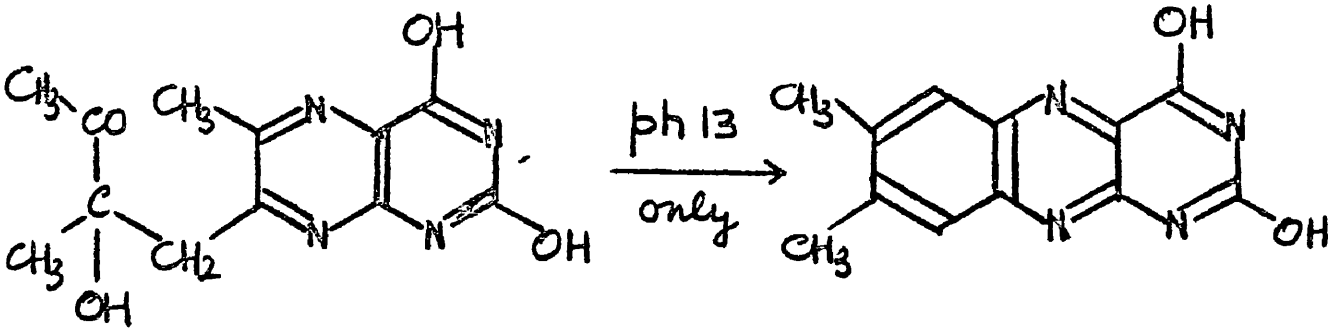
CXX

SUMMARY

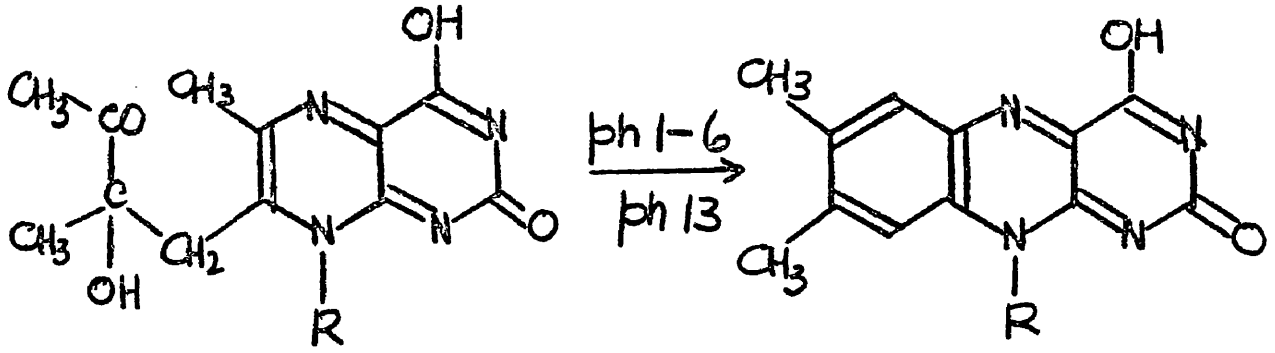
Two important observations can be made on the results detailed in Part II of this thesis. The first is that the reaction of diacetyl trimer with 5-amino-2,6-dihydroxy-4-D-ribitylaminopyrimidine to give "G-compound" and the pteridine (CXI, R = D-ribityl) may provide the link between the two separate theories of the biosynthesis of riboflavin proposed by Masuda[62] and Birch.[51,61] The early high specific activity[56,58] of labelled "G-compound" isolated from A.gossypii compared with the riboflavin produced would now be quite consistent with it not being an intermediate, and the non-conversion of "G-compound" to riboflavin using growing cultures of E.ashbyii[43] or A.gossypii[58] would be a fully expected result.

The second observation is that is strikingly evident that nitrogen 8 of the pteridine must be substituted before pteridines of the side-chain type will cyclise to give a three ringed nucleus using the dilute acid cyclisation developed during the course of this study. In other words using the pteridine (CV) cyclisation to lumichrome (CVII) proceeds only at strongly alkaline pH values, but using the pteridines (CXI, R = alkyl or sugar) cyclisation to the isoalloxazine (CXIV, R = alkyl or sugar) is effected throughout the range of pH values 1-6 and 13. This greater ease of cyclisation of N-8 substituted pteridines to isoalloxazines probably explains the inhibitive effect on

flavinogenesis of nearly all the unsubstituted diaminopyrimidines used in tests involving E.ashbyii.[42]



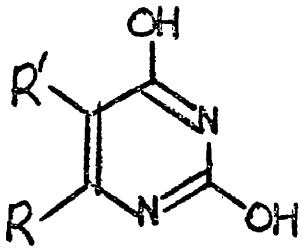
CVI CVII



CXI CXIV

TABLE 2.

Compound



λ max. (mu) (ε in parentheses) in H_2O at pH given.

R = Cl, R' = H	220(8,600),	278(10,300)	pH13
R = $N(CH_3)_2$, R' = H	220(23,300),	270(23,300)	pH13
R = NH.CH_2.CH_2OH, R' = H	220(25,700),	266(21,600)	pH13
R = NH.CH_2.CH_2OH, R'= NO	216(8,300),	310(18,000)	pH13
R = Cl, R' = NO_2	281(8,000),	315(8,600)	pH1
R = NH_2, R' = NO_2	223(19,000),	314(12,700)	pH1
	226(11,600),	334(14,900)	pH13
R = OH, R' = NO_2	218(15,800),	318(7,800)	pH1
	220(19,100),	330(10,500)	pH13
R = NH.CH_2CH_2OH, R'=NO_2	228(23,000),	324(13,400)	pH1
	216(17,900),	333(11,500)	pH13
R = NH.CH_2CH_2OAc, R'= NO_2	227(23,500),	321(12,800)	pH1
	218(16,250),	337(15,250)	pH13
R = $NHCH_3$, R' = NO_2	224(19,000),	322(11,600)	pH1
	218(15,700),	338(14,200)	pH13
R = NH.CH_2.$(CHOH)_3$.CH_2OH, R' = NO_2	228(25,700),	323(14,200)	pH1
	217(17,900),	336(17,000)	pH13

TABLE 2 (Contd.)

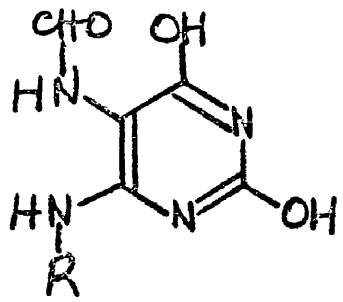
Compound	λ max.(mu) (ε in parentheses) in H_2O at pH given		
R = $N(CH_3)_2$, R' = NO_2	240(13,400),	346(4,800)	pH1
	225(13,000),	356(9,200)	pH13
R = CH_2CH_2OCHO	221(22,500),	268(17,700)	pH13
R = CH_3	218(21,500),	266(17,900)	pH13

TABLE 3

Compound	λmax.(mu) (ε in parentheses) in H_2O at pH given			
CX, R = CH_2CH_2OH	256(14,700),	276[a](10,100),	408(11,300)	pH1
	230(18,600),	280(10,700),	312(14,200)	pH13
CXV, R = CH_3		281(13,800),	324(14,600)	pH1
	220(10,700),	256(6,200),	286(7,600)	pH13
		358(10,700),		
R = CH_2CH_2OH		281(10,500),	326(10,600)	pH1
	221(12,100),	259(7,100),	288(6,500)	pH13
		356(11,000),		
CVI		244(13,200),	360(7,000)	pH13
CV		248(18,700),	362(6,900)	pH13
CXIV, R = CH_3		222(25,900),	265(27,800)	pH1
		375(8,800),	440(9,100)	
		220(36,600),	269(33,700)	pH13
		353(9,100),	444(9,300)	
R= CH_2CH_2OH		223(31,100),	267(29,600)	pH1
		376(9,900),	444(10,900)	
		222(27,700),	270(35,800)	pH13
		356(11,700),	446(12,300)	
R = D-ribityl		223(35,500),	267(35,500)	pH1
		376(10,700),	445(11,500)	
		222(26,600),	270(31,700)	pH13
		356(10,600),	447(10,600)	
CXII, R = CH_3	260(21,300),	298[a](10,200),	434(12,000)	pH1
	226(22,300),	272(18,300),	456(16,100)	pH13
R.CH_2CH_2OH	260(16,200),	300[a](9,000),	430(10,900)	pH1
	224(19,200),	272(15,800),	454(14,500)	pH13

a = shoulder

TABLE 4

Compound	λ max. (mu) (ε in parentheses) in H_2O at pH given			
CXX, R = CH_3	222(25,100)	277(10,900),	418(24,000)	pH1
	236(32,700),	272(14,400),	431(35,500)	pH13
, R=CH_2CH_2OH	228(20,400),	278(9,300),	422(20,800)	pH1
	231(29,100),	272(13,100),	436(31,400)	pH13
R = D-ribityl	230(22,600),	280(10,300),	422(22,200)	pH1
	230(27,100),	272(11,900),	436(26,600)	pH13
Self-Condensation				
Methyl Series	222(21,300),	276(9,600),	416(20,000)	pH1
	238(25,600),	278(9,200),	430(25,800)	pH13
β-hydroxy ethyl series	228(21,000),	280(9,300),	422(22,300)	pH1
	232(28,200),	272(12,100),	437(29,700)	pH13
D-ribityl series	232(29,000),	280(12,700),	422(31,200)	pH1
	232(25,400),	276(10,000),	434(30,900)	pH13

EXPERIMENTAL

2,4,6-Trihydroxypyrimidine ("barbituric acid")[109]. - Ethyl malonate (80 g.) and urea (30 g.) in absolute ethanol (250 c.c.) were added to sodium ethoxide (11.5 g. of sodium in 250 c.c. absolute ethanol) and the mixture was refluxed for 7 hr. in an oil-bath at 100-110°. Hot water (500 c.c. 50°) was then added followed by 12N-hydrochloric acid (47 c.c.). After stirring the solution was filtered and left to stand overnight at 0°. The crystalline product was collected, washed with cold water (50 c.c.), and oven-dried at 100-110° for 2 hr. to give off-white crystals (50 g., 78%), m.p. 240° (decomp.) [lit.,[109] m.p. 240°]

2,4,6-Trichloropyrimidine.[97] - 2,4,6-Trihydroxypyrimidine (50 g.) was added portion-wise over 10 min. to a mixture of diethylaniline (95 c.c.) and phosphoryl chloride (156 c.c.), after which the whole was refluxed for 5 min., cooled, and poured on ice (800 g.). The mixture was extracted with ether (750 c.c.) and the ether extract was dried over anhydrous sodium sulphate. The ether was removed by evaporation *in vacuo* and the oily residue was subjected to fractional distillation at reduced pressure, the fraction of b.p.110° being collected to give the trichloropyrimidine (48 g., 66%) as a clear viscous oil with extreme vesicant properties.

4-Chloro-2,6-dihydroxypyrimidine ("4-chlorouracil").[98] - 2,4,6-Trichloropyrimidine (48 g.) was refluxed for 16 hr. in a solution of sodium hydroxide (41.8 g.) in water (420 c.c.). On cooling the sodium salt of 4-chloro-2,6-dihydroxypyrimidine separated out, and this was converted to the free pyrimidine by the addition of 12N hydrochloric acid (50 c.c.). The product was collected, washed with water, and dried over silica gel, to give the pyrimidine as a white powder (35.2 g., 92%), m.p. 300° (decomp.), showing one spot on paper chromatography.

Reaction of 4-Chlorouracil and Ethanolamine in Dimethyl Formamide. - 4-Chlorouracil (3 g.) and ethanolamine (3 g.) were refluxed together for 2 hr. in dimethyl formamide (50 c.c.) On cooling a white product (0.275 g.) was precipitated and collected, the filtrate on reduction of volume gave a further crop (0.880 g.). The two crops were bulked and twice recrystallised from water to give white plates (0.75 g.), m.p.>300°. (Found: C,46.4; H,5.2; N,27.5. Calc. for $C_6H_9N_3O_2$. C,46.4; H,5.8; N,27.1%.

4-Dimethylamino-2,6-dihydroxypyrimidine.- (a) 4-Chlorouracil (0.5 g.) and dimethylamine (3 c.c. of a 33% w/w alcoholic solution) were heated at 120° for 4 hr. in ethanol (25 c.c.) in a sealed tube. The resulting solution was taken to dryness and the residue was recrystallised three times from water to give white plates (0.375 g.) of 4-dimethylamino-2,6-dihydroxypyrimidine, m.p.>300°

(lit.,[110] m.p. > 300°) which was identical with the product of reaction immediately above in infrared absorption spectrum and on paper chromatography. (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 (0.5 g.) and dimethyl formamide (25 c.c.) were refluxed together for 2 hr. The product was shown by paper chromatography and light absorption to be identical with 4-dimethylamino-2,6-dihydroxypyrimidine.

4-β-Hydroxyethylamino-2,6-dihydroxypyrimidine. - 4-Chorouracil (2 g.) and ethanolamine (2 g.) in water (50 c.c.) were heated together at 140° for 2 hr. in a sealed tube. The water was removed *in vacuo* and the residue thrice recrystallised from water to give white needles (0.31 g.) of 4-β-hydroxyethylamino-2,6-dihydroxypyrimidine, 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%).

4-β-Hydroxyethylamino-2,6-dihydroxy-5-nitrosopyrimidine. - 4-β-Hydroxyethylamino-2,6-dihydroxypyrimidine (0.13 g.) was dissolved with heating in 2N-hydrochloric acid (3 c.c.) and the solution was chilled in a freezing mixture. To the chilled solution was added, dropwise, sodium nitrite (0.16 g.) in water (1.6 c.c.). The mixture was allowed to come to room temperature, and the pink solid (0.133 g.) was collected. The product was twice recrystallised from water to give 4-β-hydroxyethylamino--2,6-dihydroxy-5-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%).

D-Ribose oxime.[99] - Sodium ethoxide (1.16 g. of sodium in 25 c.c. of absolute ethanol) was added to a solution of dry hydroxylaminehydrochloride (3.6 g.) in absolute ethanol (75 c.c.) and the mixture was allowed to stand overnight. After removal, by filtration, of sodium chloride the filtrate was heated to 70° and the hot solution treated portion-wise with D-ribose (5 g.). The mixture was allowed to stand overnight at room temperature after which the product was collected, washed with ethanol and then ether, to give D-ribose oxime (5.1 g., 93%) as white needles, m.p. 139-141° (lit.[99] 140°).

Acetylation of D-ribose Oxime. - D-ribose oxime (1.5 g.) was dissolved in pyridine (12 c.c.) and treated with acetic anhydride (9 c.c.) at room temperature. After 3 hr. the mixture was poured into water (100 c.c.) and the aqueous solution was extracted with chloroform (200 c.c.). The chloroform layer was washed first with 4N-hydrochloric acid and then with water, after which it was dried over anhydrous sodium sulphate. Removal of the chloroform in vacuo gave a clear intractible gum (3.12 g., 94%) whose infrared absorption spectrum showed acetate and oxime bands, but no hydroxyl or nitrile bands.

D-ribitylamine.[94] - PtO_2 (0.5 g.) was suspended in water (100 c.c.) and reduced with hydrogen. A solution of D-ribose oxime (1 g.) in water (30 c.c.) was then added dropwise, and the whole hydrogenated until the theoretical uptake was obtained.

After removal of the catalyst the solution was made up to 250 c.c. and was shown to contain amine (0.876 g. based on D-ribitylamine, 95.7%) by titration with $N/_{10}$ hydrochloric acid to the methyl-red end-point.

Oxalate of D-ribitylamine. - D-ribose oxime (1 g.) was reduced as above and the resulting amine solution was run on to a column of Amberlite IRC50 ion-exchange resin (1.5 x 15 cm., H^+ form). The column was washed with water (300 c.c.) and the product eluted with 0.1M oxalic acid. The eluate was collected in 10 c.c. fractions which were tested for acidity and colouration with ninhydrin. Those fractions which were both neutral and positive to ninhydrin were examined in paper chromatography the papers being developed using periodate solution followed by Schiff's reagent. By this method the eluate of the column was shown to consist of at least two amines.

4-Amino-2,6-dihydroxy-5-nitropyrimidine. - 4-Chlorouracil (3.5 g.) was dissolved gradually in 36N sulphuric acid (11 ml.) at $<45°$. Nitric acid (d, 1.5; 3.8 ml.) was slowly added with stirring maintaining the temperature at 0°. The solution was then kept at room temperature for 30 min. until a solid was precipitated. The mixture was then treated with ice (80 g.) and the pH of the solution was adjusted to pH7 by the addition of 10N ammonium hydroxide. Recrystallisation of the collected product gave 4-amino-2,6-dihydroxy-5-nitropyrimidine as a white crystalline solid (2 g.) m.p. $>330°$ (lit.[111], m.p. $>330°$) (Found:

C,25.6; H,3.2. Calc. for $C_4H_4N_4O_4.H_2O$. C,25.3; H,3.2%).

4-Chloro-2,6-dihydroxy-5-nitropyrimidine. - 4-Chlorouracil (5 g.) was dissolved gradually in 36N sulphuric acid (6 ml.) at $<45°$. Nitric acid (d,1.5; 5.3 ml.) was slowly added with stirring at 0°. The solution was then kept at room temperature for 30 min. until solid precipitated, after which it was treated with ice (20 g.). The white product was collected, washed with water (2 x 20 ml.), and dried *in vacuo* over phosphorus pentoxide Yield 2.35 g., m.p. 220-222°. (Found: C,25.8; H,1.5. $C_4H_2N_3O_4Cl$ requires C,25.1; H,1.1%).

2,4,6-Trihydroxy-5-nitropyrimidine. - The filtrate of the immediately above reaction after removal of 4-chloro-2,6-dihydroxy--5-nitropyrimidine was diluted with water to 100 ml. and left overnight at room temperature. The white crystalline product was collected and dried to give 2,4,6-trihydroxy-5-nitropyrimidine (1.6 g.), m.p. 180° (lit.[110], 181°) identical with an authentic sample of this compound in paper chromatography, and ultraviolet and infrared absorption, and showing no depression on taking a mixed melting-point.

4-β-Hydroxyethylamino-2,6-dihydroxy-5-nitropyrimidine. - 4-Chloro-2,6-dihydroxy-5-nitropyrimidine (1 g.) in ethanol (50 c.c.) was treated with ethanolamine (2 equivalents). The mixture was then heated to boiling and treated with water drop-wise until all the product just dissolved. On cooling 4-β-hydroxyethylamino-2,6-dihydroxy-5-nitropyrimidine separated out as fine white needles

(1.08 g.), m.p. 217-219°.(Found: C,33.6; H,3.8; N,26.2. $C_6H_6N_4O_5$ requires C,33.3; H,3.7; N,26.0%).

4-β-Acetoxyethylamino-2,6-dihydroxy-5-nitropyrimidine.- 4-β-Hydroxyethylamino-2,6-dihydroxy-5-nitropyrimidine (1 g.) was treated with pyridine (10 c.c.) and acetic anhydride (16 c.c.), and the mixture was heated for 20 min. on a steam bath. After standing overnight at 0° the mixture was filtered and the product recrystallised from water to give the acetate (.5 g.) as white needles, m.p. 326°. The filtrate, after destruction of excess acetic anhydride with ethanol, was reduced in volume in vacuo to give a further crop of the acetate (0.3 g.) which after one recrystallisation from water also melted at 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%).

4-Methylamino-2,6-dihydroxy-5-nitropyrimidine. - 4-Chloro-2,6--dihydroxy-5-nitropyrimidine (1.3 g.) in ethanol (60 c.c.) was treated with methylamine (2 equivalents) in alcoholic solution (33% w/w). The mixture was heated to boiling and treated with just enough water to effect solution. On cooling 4-methylamino--2,6-dihydroxy-5-nitropyrimidine separated out as lustrous white needles (1.1 g.), m.p. $>330°$. (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-5-nitro-4-D-ribitylaminopyrimidine. - D-Ribose oxime (6.6 g.) was hydrogenated using PtO_2 (2.6 g.) as catalyst according to the procedure reported above. When the hydrogenation was finished the catalyst was removed and the amine solution

treated with a solution of 4-chloro-5-nitrouracil (3.9 g.) in ethanol (200 c.c.) at room temperature. The resulting mixture was left to stand for 24 hr. at room temperature and the solid product (1 g.) was then collected. This product was identical with 4-amino-2,6-dihydroxy-5-nitropyrimidine when compared with an authentic sample of this pyrimidine using infrared and ultraviolet absorption spectroscopy, and paper chromatographic techniques.

The filtrate which still contained two other pyrimidines by examination using paper chromatography was treated with ammonia and formic acid until the solution was buffered at pH10.7. This solution was next run on to a column of the anion exchange resin (Amberlite CG 400; formate form) which had been previously prepared by washing with ammonium formate buffer (M/10 with respect to formic acid) at pH 10.7. When all the pyrimidine solution had been absorbed on the column thorough washing with the ammonium formate buffer (M/10 with respect to formic acid; pH10.7) was commenced. No product was eluted at this stage. The pH of the buffer solution (still M 10 with respect to formic acid) was now changed to 7.4 and a bright yellow pyrimidine was eluted from the column in a fairly broad band. This pyrimidine shows ultraviolet absorption maxima at 322mu at pH1, and 334mu at pH13, and is as yet of unknown constitution.

The buffer solution was next changed to one of pH4 (M/10 with respect to ammonia) and the eluate soon gave a pyrimidine in a

narrow band. All fractions containing this pyrimidine, checked by paper chromatography and ultraviolet absorption spectroscopy, were bulked and the water was reduced in volume *in vacuo* at a temperature not exceeding 40°. When only about 10 c.c. of aqueous solution remained ethanol (500 c.c.) was added and the resulting white precipitate was collected. The pyrimidine was freed of any ammonium formate by boiling in ethanol (150 c.c.) in which ammonium formate is soluble. The *white pyrimidine* (2.7 g.) could not be crystallised, m.p. 203-204°. (Found: C,33.8; H,4.8; N,17.4. $C_9H_{14}N_3O_3$ H_2O requires C,33.3; H,5.0; N,17.3%) $[\alpha]_D^{23}$ + 4.5° in N_{10} sodium hydroxide (*c*,1.125).

The ribitylaminopyrimidine showed an uptake of 2.7 mol. of periodic acid on oxidation.

2,6-*Dihydroxy*-4-*dimethylamino*-5-*nitropyrimidine*. - 4-Chloro--2,6-dihydroxy-5-nitropyrimidine (1 g.) in ethanol (50 c.c.) was treated with a solution of dimethylamine in methanol (33% w w) just to alkaline reaction with narrow-band indicator paper (B.D.H. pH7-8.5). After 15 min. at room temperature the product was collected and recrystallised from ethanol to give the *pyrimidine* as bright yellow needles (0.8 g.), m.p. 186-189° (Found: C,36.2; H,3.9; N,28.4. $C_6H_5N_4O_4$ requires C,36.0; H,4.0; N,28.0%).

Preparation of Anion Exchange Resins for Aldol Condensations of Diacetyl.[113] - The resins used for the purpose were Amberlite CG 400 and Amberlite IRA 400. Both were prepared by washing with

5% sodium hydroxide solution until the eluate gave a negative test for chloride ion. The resins were then washed with water till free of all excess sodium hydroxide and dried overnight at 30°. The resins were used within 24 hr of drying.

Diacetyl Dimer (5-acetyltetrahydro-2-hydroxy-2,5-dimethyl-3-oxo furan).[51,103] (a) During a period of 1 hr. N-potassium hydroxide solution (350 c.c.) was allowed to drop into a stirred solution of diacetyl (100 g.) in water (300 c.c.) while the reaction mixture was maintained at 0°. The pale yellow solution after acidification with 10% sulphuric acid was extracted with ether (750 c.c.). The ethereal extract after drying over anhydrous sodium sulphate was taken to dryness *in vacuo* and so gave a yellow viscous liquid (60 g.). The liquid was fractionally distilled at 89°/0.2 mm. and this procedure gave a pale yellow gum which could not be crystallised. The product had boiling point 89° at 0.2 mm. (Diels, Blanchard and D'Heyden give 89° at 0.2 mm.) and its infrared absorption spectrum showed peaks at 3,576, 1720 and 1770 $cm.^{-1}$ (Birch and Moye give 3,600, 1720 and 1770 $cm.^{-1}$).

(b) Diacetyl (30 c.c.) was treated portion-wise with the anion exchange resin (Amberlite CG 400; OH form ; 10 g.) and the mixture was shaken and left to stand overnight at room temperature. During the mixing procedure a slight increase in the temperature of the reaction was allowed, but any tendency for

the reaction to become vigorous was checked. The resulting paste was treated with ether (100 c.c.) and the ion-exchange resin was removed by filtration. Removal of the ether then gave a faint yellow liquid (19 g.) which was identical to that obtained by procedure (a) on infrared spectral comparison.

Diacetyl Trimer. (a) Diacetyl (30 c.c.) was treated portion-wise with the anion exchange resin (Amberlite CG 400; OH form; 10 g.) as in the preparation (b) of diacetyl dimer, except that on this occasion the reaction was slowed down by rigid control of the temperature. When the reaction mixture became very viscous (3-5 days) it was treated with ether (100 c.c.) and the exchange resin was filtered off. Removal of the ether gave a light yellow gum to which water (3 c.c.) was added and the whole refrigerated overnight. The resulting crystals were collected and recrystallised from ether to give white needles (2 g.), m.p. 66-75°.

The compound had a molecular weight of 251 (elevation of the boiling point of benzene) which indicated that it was a trimer of diacetyl (M.W.258). X-Ray crystallographic studies showed the crystal to be monoclinic and showed the unit cell to contain 12 diacetyl units, and this result also suggests a trimer. The elementary analysis also indicated a polymer of diacetyl (Found: C, 55.9; H, 7.2. $(C_4H_6O_2)_x$ requires C, 55.8; H, 7.0%).

The trimer shows no absorption in the ultraviolet, but absorbs in the infrared where it has a very complex spectrum with a very

strong carbonyl band at 1706 $cm.^{-1}$ (open chain ketone) and bands at 3436 $cm.^{-1}$ (OH) and 2976 $cm.^{-1}$ ($C-CH_3$). The N.M.R. spectrum of the trimer indicates that there is only one aldol linkage in its formation.

(b) Diacetyl (25 c.c.) was treated with the anion exchange resin (IRA 400; OH form; 8 g.) and the mixture was left at room temperature until crystals separated out and the whole became solid (3-5 days). The resin was removed by solution of the crystalline product in ether (200 c.c.) followed by filtration. Removal of the ether gave a crystalline mass, which on recrystallisation (twice) from ether gave white needles (6.5 g.), m.p. 70-77°, identical on infrared spectral comparison with the product of preparation (a).

Treatment of Diacetyl Trimer with Periodic Acid. - (a) The trimer of diacetyl (0.4954 g.) was treated with sodium metaperiodate (25 c.c. of a solution containing 5.3996 g./100 c.c.) and the volume was made up to 50 c.c. After 4 hr. the periodate content of a 5 c.c. aliquot of this solution was determined using 0.1042N sodium arsenite solution. A blank solution similar to the above but without any trimer was made up, and the periodate content of a 5 c.c. aliquot of it was likewise estimated.

The positive aliquot required 4.8 c.c. of sodium arsenite, and the blank aliquot required 12 c.c. of sodium arsenite. These results show an uptake of periodate equivalent to 1.96 moles per mole of trimer.

(b) The above procedure was repeated except that on this occasion titrations were made after 2 hr., 4 hr., 6 hr., and 24 hr. These showed uptakes of periodate equivalent to 1.75, 1.93, 1.98 and 2.05 moles per mole of trimer respectively.

(c) Diacetyl trimer (0.2572 g.) was treated with exactly 2 moles of sodium metaperiodate (10 c.c. of a solution containing 4.8664 g./100 c.c.) and the solution was made up to 25 c.c. after 4 hr. a 5 c.c. aliquot of the solution was titrated and the periodate uptake was shown to be 1.95 moles/mole of trimer. The remaining 20 c.c. of the solution were treated with ethylene glycol (0.1325 g.) and after a further ½ hr. with dimedone (1.0109 g.). The mixture was heated for 10 min. at 90° and so gave the formaldehyde-dimedone complex. The solid was collected and dried to give a white powder (0.7662 g.; 73% based on initial sodium periodate present) m.p. 190° (lit.,[75] 189-190°).

2,4-Dihydroxy-6,7-dimethylpteridine.[51,105] - 4,5-Diamino-2,6-dihydroxypyrimidine sulphate (2.5 g.) in water (80 c.c.) was treated with diacetyl (2 equivs.). The mixture was heated on a steam bath until everything had gone into solution, after which it was left overnight at 0°. The product was collected, washed with water, and dried at 100° to give the pteridine as colourless needles (1.6 g.), m.p. 340° (lit.,[51] 340°).

7-(2'-Hydroxy-2'-methyl-3'-oxobutyl)-2,4-dihydroxy-6-methylpteridine. - (a) 4,5-Diamino-2,6-dihydroxypyrimidine sulphate

(0.5 g.) in water (25 c.c.) was treated with the diacetyl dimer (2 equivs.). The mixture was heated until complete solution, treated with charcoal, filtered, and left at 0°. 7-(2'-Hydroxy-2'-methyl-3'-oxobutyl)-2,4-dihydroxy-6-methylpteridine thus precipitated as golden plates which were twice recrystallised (charcoal) from water to give light yellow crystals, (0.26 g.), m.p. 226-228°. Further recrystallisation gives m.p. 230-232° (lit.,[51] 230-232°). (Found: C,51.5; H,5.2. Calc for $C_{12}H_{14}N_4O_4$. C,51.8; H,5.1%).

(b) Diacetyl trimer (1.85 g.) was dissolved in water (40 c.c.) by heating on a steam-bath, and 4,5-diamino-2,6-dihydroxypyrimidine sulphate (2.45 g.) was added. Heating was continued to complete solution, charcoal was added, the solution was boiled for a few minutes and then rapidly filtered. After standing overnight at 0° the off-white product was collected and twice recrystallised from water, using charcoal, to give the pteridine as light yellow prisms, (0.6 g.), m.p. 227-229°. This product was identical with that obtained in method (a) in paper chromatography, ultraviolet and infrared spectra, and in its subsequent convertibility to lumichrome (below).

Further examination of the mother liquors of this reaction, both by paper chromatography and ultraviolet spectroscopy, showed that they contained 2,4-dihydroxy-6,7-dimethylpteridine.

2,4-Dihydroxy-7,8-dimethyl-benzo[g]pteridine ("lumiohrome").

(a) The pteridine (CV, 0.35 g.) was heated at 100° in N-sodium

hydroxide solution (35 c.c.) for 1 hr. On cooling the sodium salt of lumichrome was deposited. The mixture was acidified with 2N-hydrochloric acid and the gelatinous yellow lumichrome (0.29 g.) was collected, washed with water and dried, m.p. $>300°$ (lit.,[115] $>300°$).

The ultraviolet absorption spectrum of the product at pH13 showed peaks at 222, 260, 340, and 428 mu, which is identical with that of an authentic specimen of lumichrome.

(b) The procedure in (a) was repeated using $N/_{10}$-sodium hydroxide in place of N-sodium hydroxide and the product was again shown by ultraviolet spectroscopy and paper chromatography to be lumichrome.

(c) The procedure in (a) was repeated using $N/_{10}$-hydrochloric acid in place of N-sodium hydroxide. On this occasion heating was continued for 6 hr. On cooling the pteridine starting material was recovered, and paper chromatography of the reaction mixture showed no traces of lumichrome.

Di-formyl Derivative of 5-amino-2,6-dihydroxy-4-β-hydroxy ethylaminopyrimidine. - 2,6-Dihydroxy-4β-hydroxyethylamino-5--nitropyrimidine (0.5 g.) was dissolved in 98% formic acid (20 c.c.) and the solution was treated with zinc dust (0.6 g.). The mixture was refluxed for 8 hr. and the zinc salts were removed by filtration of the still hot solution. Immediate addition of ethanol (25 c.c.) and ether (200 c.c.) to the filtrate gave a white precipitate (0.4 g.), which was collected, washed with ether, and freed of any traces of formic acid by standing over sodium

hydroxide pellets *in vacuo*. Recrystallisation from water gave white needles (0.22 g.) m.p. > 325°. (Found: C,39.7; H,4.3; N,23.3. $C_8H_{10}N_4O_5$ requires C,39.7; H,4.2; N,23.1%).

The Formyl Derivative of 5-Amino-2,6-dihydroxy-4-methylamino-pyrimidine. - 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0.33 g.) was dissolved in 98% formic acid (20 c.c.) and the solution was treated at 90° with zinc dust (0.5 g.). After the initial reaction had subsided the mixture was heated at 90° for 10 min. and then filtered hot to remove zinc salts. Immediate addition of alcohol and ether to the filtrate precipitated the pyrimidine as a white solid (0.25 g.). Recrystallisation of the white solid from water gave the formyl compound as long white needles (0.19 g.) m.p. > 325° (Found: C,39.0; H,4.4; N,30.1. requires C,39.1; H,4.4; N,30.4%).

2,8-Dihydro-4-hydroxy-8-β-hydroxyethyl-6,7-dimethyl-2-oxo-pteridine. - (a) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitroso-pyrimidine (0.3 g.) in water (50 c.c.) was hydrogenated using a palladium charcoal catalyst (10% palladium; 0.15 g.). When the uptake reached theoretical (20 min.) the hydrogen in the apparatus was replaced by nitrogen and the solution was treated with diacetyl (0.3 g.; 100% excess) in alcohol (25 c.c.). After 30 min. the catalyst was removed by filtration and the bright green fluorescing solution was reduced in volume at less than 40° to *circa* 10 c.c. and refrigerated. The resulting crystals were collected and twice recrystallised from water to give the

oxo-pteridine as dark-orange needles (0.05 g.), m.p. 270° (dec.) (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%).

(b) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.4 g.) in water (50 c.c.) was hydrogenated using platinum oxide (0.2 g.) as catalyst. After the theoretical uptake was reached (5 hr.) the reaction mixture was treated as in procedure (a) and gave the oxopteridine as orange needles (0.08 g.), m.p. 270° (dec.). The product was identical with that obtained in procedure (a) on comparison using ultraviolet and infrared spectroscopy, and using paper chromatography.

(c) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.5 g.) in water (75 c.c.) was hydrogenated using platinum oxide (0.2 g.) as catalyst in the manner of procedure (b). On this occasion, however, when the hydrogenation was complete diacetyl trimer (0.5 g.) was added in place of diacetyl. The work-up was then as in (a) and (b) and the initial crystalline product (0.42 g.) was twice recrystallised from water to give bright orange needles (0.075 g.), m.p. 270° (dec.). This product was shown to be identical with those of procedures (a) and (b) by all the normal identification techniques.

Reaction of Diacetyl Dimer with 5-Amino-2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine. - (a) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.5 g.) was dissolved in water (10 c.c.) and made alkaline with 10% sodium hydroxide (3 c.c.). The solution was reduced with sodium dithionite in the usual manner and after the

finish of the reaction the pH was adjusted to 4 by the addition of 12N-hydrochloric acid. The solution was then treated with diacetyl dimer (0.5 g.) and the mixture was heated for 15 min. at 90°. The orange solution was examined in paper chromatography using solvent system (A) and was shown to consist of two spots. The one of lower R_F value exhibited a bright yellow green fluorescence and had the same ultraviolet absorption spectrum as 2,8-dihydro-4-hydroxy-8-β-hydroxyethyl-6,7-dimethyl-2-oxopteridine both in acid and alkali, but its R_F value did not agree with that of the latter compound. The more mobile component exhibited an orange fluorescence and had a new type of ultraviolet absorption spectrum.

The reaction mixture was separated using paper chromatography on sheets of thick filter paper (Whatman No. 17) and solvent system (A). The bands from several sheets corresponding to the orange and the yellow green fluorescing compounds were cut from the dried paper and treated with water (total volume 400 c.c. for each). When the compounds had been fully extracted from the paper, the paper was removed by filtration and the volume of each filtrate was reduced at low temperature <u>in vacuo</u> to about 10 c.c. The yellow-green fluorescing solution gave a very hygroscopic orange solid which rapidly underwent decomposition to give a dark-brown gum. The orange fluorescing solution deposited bright orange crystals (0.025 g.), which recrystallised from water as needles, m.p. 266-268°. (Found: C, 54.6; H, 5.2; $C_{14}H_{16}N_4O_4$ requires C, 55.2; H, 5.3%).

(b) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.25 g.) was dissolved in water (10 c.c.) and was reduced using sodium dithionite in alkaline solution as above in (a). The reduced solution was taken to pH4 by the addition of 12N-hydrochloric acid, and treated with diacetyl dimer (0.25 g.). After heating for 15 min. at 90° the pH was adjusted to 7 using the required amount of sodium hydrogen carbonate, and the reaction mixture treated with enough N-sodium hydroxide solution to give an overall normality of 0.1N. Heating was continued at 90° for a further 1 hr. at the end of which the reaction mixture was taken to pH1 using 12N-hydrochloric acid and placed in the refrigerator. Orange crystals (0.025 g.) of 6,7-dimethyl-9-β-hydroxyethyliso alloxazine deposited overnight, m.p. 300° (lit.,[114] 300-301°).

(c) The di-formyl derivative of 5-amino-2,6-dihydroxy-4-β--hydroxyethylaminopyrimidine (0.5 g.) was treated with diacetyl dimer (1 g.) and 0.1N-hydrochloric acid (10 c.c.) and the mixture was heated for 8 hr. at 90°. The resulting orange product was collected and recrystallised from water to give the 9-β-hydroxy-ethylisoalloxazine as pure orange needles (0.06 g.) m.p. 300-301°.

(d) The diformyldderivative of 5-amino-2,6-dihydroxy-4-β-hydroxy ethylaminopyrimidine (0.22 g.) was treated with diacetyl dimer (0.5 g.) and N-hydrochloric acid (15 c.c.). The mixture was refluxed for 30 min., cooled and filtered to give 6,7-dimethyl--9-β-hydroxyethylisoalloxazine as pure orange needles (0.04 g.) m.p. 301°.

(e) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.36 g.) in hot water (8.5 c.c.) was treated with zinc dust (1.2 g.) and 10N-sulphuric acid (1.1 c.c.). The mixture was refluxed for 10 min. and the zinc residues were then filtered from the still hot solution. Diacetyl dimer (0.75 g.) in water (5 c.c.) was added to the filtrate and the reaction mixture was refluxed for 15 min. The mixture was once more filtered, to remove impurities, and the filtrate was adjusted to pH1 using 12N-hydrochloric acid. Refluxing of the reaction mixture for a further 15 min., followed by cooling, gave 6,7-dimethyl-9-β-hydroxyethylisoalloxazine as pure microneedles (0.1 g.) m.p. 298-300°.

Reaction of Diacetyl Trimer with 5-amino-2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine. - (a) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.25 g.) was dissolved in water (10 c.c.) and reduced with sodium dithionite in alkaline solution in the usual manner. The reduced solution was taken to pH4 by the addition of 12N-hydrochloric acid, and treated with diacetyl trimer (0.25 g.). The mixture was heated for 10 min. at 90° after which it was examined using paper chromatography and ultraviolet absorption spectroscopy. By these means the mixture was shown to consist of three compounds, the orange fluorescing and yellow-green fluorescing compounds encountered in the reaction with diacetyl dimer (see above) plus a further yellow-green fluorescing compound which was shown to be 2,8-

-dihydro-4-hydroxy-8-β-hydroxyethyl-6,7-dimethyl-2-oxopteridine.

(b) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.25 g.) was dissolved in water (10 c.c.) and was reduced using sodium dithionite in alkaline solution in the usual manner. The reduced solution was taken to pH4 by the addition of 12N-hydrochloric acid, and treated with diacetyl trimer (0.4 g.). After heating for 15 min. at 90° the pH was adjusted to 7 using the required amount of sodium hydrogen carbonate, and the reaction mixture treated with enough N-sodium hydroxide solution to give an overall normality of 0.1N. Heating was continued at 90° for 1 hr. and the reaction mixture was then refrigerated. No solid separated out, but the mixture was shown to contain the 9-hydroxyethylisoalloxazine both by paper chromatography and by ultraviolet absorption spectroscopy.

(c) The di-formyl derivative of 5-amino-2,6-dihydroxy-4-β--hydroxyethylaminopyrimidine (0.1 g.) was treated with diacetyl trimer (0.25 g.) and 0.1N-hydrochloric acid (10 c.c.) and the mixture was heated for 8 hr. at 90°. The resulting orange crystals (0.03 g.) were recrystallised from water to give 6,7-dimethyl-9-β-hydroxyethylisoalloxazine as orange needles, (0.02 g.) m.p. 300°.

(d) The di-formyl derivative of 5-amino-2,6-dihydroxy-4-β--hydroxyethylaminopyrimidine (0.15 g.) was treated with diacetyl trimer (0.15 g.) and N-hydrochloric acid (10 c.c.). The mixture was refluxed for 30 min, and then cooled so giving 6,7-dimethyl -9-β-hydroxyethylisoalloxazine as pure orange needles (0.015 g.), m.p. 299-300°.

The mother liquors of reactions (b), (c) and (d) above all contained considerable quantities of 2,8-dihydro-4-hydroxy-8-β-hydroxyethyl-6,7-dimethyl-2-oxopteridine.

Reaction of Diacetyl Dimer with 5-Amino-2,6-dihydroxy-4-methylaminopyrimidine. - (a) 2,6-Dihydroxy-4-methylamino-5--nitropyrimidine (0.25 g.) was dissolved in water (10 c.c.) and the solution was treated with 10% sodium hydroxide (5 c.c.). The nitro group was next reduced to an amino group as above using sodium dithionite, and after adjustment of the pH of the reduced solution to 4 diacetyl dimer (0.5 g.) was added. The mixture was heated at 90° for 20 min. after which the pH was raised to 7 using sodium hydrogen carbonate. Sufficient sodium hydroxide was next added to give a normality of N_{10} based on the total volume of the mixture. The mixture was further heated at 90° for 1 hr. Acidification with a few drops of 12N-hydrochloric acid and extraction with chloroform (100 c.c.) gave a yellow chloroform extract which showed a bright green fluorescence and which was shown to contain lumiflavin both by paper chromatography and ultraviolet absorption spectroscopy.

(b) 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0.23 g.) was dissolved in water (10 c.c.) and the solution was treated with sodium hydroxide and sodium dithionite as in (a). When the reduction was complete the pH was adjusted to pH3.5 using 12N-hydrochloric acid. Diacetyl dimer (0.5 g.) was then added and the mixture was heated at 90° for 15 min. At the end of this

period the solution was treated with more 12N-hydrochloric acid to pH1 and heated at 90° for a further 30 min. Extraction with chloroform (75 c.c.) gave a yellow solution which on evaporation *in vacuo* gave lumiflavin as a pure orange powder (0.03 g.).

The aqueous layer from the chloroform extraction was left overnight at room temperature and gave an orange solid (0.035 g.), which was collected, and recrystallised from water to give orange needles, m.p. 258-262°. This orange compound analyses for lumiflavin plus one mole of water (Found: C,55.5; H,5.1; $C_{13}H_{14}N_4O_3 . 0.5H_2O$ requires C,55.2; H,5.3%).

Reaction of Diacetyl Trimer with 5-Amino-2,6-dihydroxy-4--methylaminopyrimidine. - 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0.2 g.) was dissolved in water (10 c.c.) and reduced as above. After completion of the reduction and adjustment of pH to 3.5 diacetyl trimer (0.28 g.) was added and the mixture was heated at 90° for 15 min. The pH was next further reduced using 12N-hydrochloric acid to pH1 and the mixture was refluxed for 1 hr. Lumiflavin separated out overnight as bright orange needles (0.04 g.), m.p. 326° (lit.,[116] 328°).

Reaction of Diacetyl Dimer and 5-Amino-2,6-dihydroxy-4-D--ribitylaminopyrimidine. - (a) 2,6-Dihydroxy-5-nitro-4-D--ribitylaminopyrimidine (0.25 g.) was dissolved in water (10 c.c.) and made alkaline with N-sodium hydroxide (5 c.c.). The nitro group was then reduced using sodium dithionite as above. When the reduction was complete the pH was adjusted to 4 by the

addition of 12N-hydrochloric acid. The solution was treated with diacetyl dimer (0.5 g.) and the mixture was heated for 15 min. at 90°. The orange solution was examined in paper chromatography using solvent system (A) and was shown to consist of two spots which were identical in fluorescence and ultraviolet absorption spectra with the two products produced by the reaction of diacetyl dimer and 5-amino-2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine.

(b) 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine (0.75 g.) was dissolved in water (30 c.c.) and reduced using sodium dithionite in alkaline solution as in (a). The reduced solution was taken to pH4 by the addition of 12N-hydrochloric acid, and treated with diacetyl dimer (0.75 g.). After heating for 10 min. at 90° the pH was readjusted to 7 using sodium hydrogen carbonate, and the reaction mixture treated with enough N-sodium hydroxide solution to give an overall normality of 0.1N. Heating was continued at 90° for a further 1 hr. at the end of which the reaction mixture was acidified. Riboflavin was shown to be the product of this cyclisation both by paper chromatography and ultraviolet absorption spectroscopy.

(c) 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine (0.2 g.) was dissolved in water (5 c.c.) and N-sodium hydroxide (2 c.c.) was added. The solution was then reduced with sodium dithionite in the usual manner. The pH was then adjusted to 1 and the solution was treated with diacetyl dimer (0.25 g.). The mixture was heated at 90° for 8 hr., and cooled to give riboflavin as an

orange powder (0.016 g.), m.p. 282°, identical with authentic riboflavin in all its physical properties.

Reaction of Diacetyl Trimer and 5-Amino-2,6-dihydroxy-4-D--ribitylaminopyrimidine. - (a) 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine (0.5 g.) was dissolved in water (15 c.c.) and made alkaline with N-sodium hydroxide (5 c.c.). Sodium dithionite was next added until the solution became colourless. The reduced solution was taken to pH4 by the addition of 12N-hydrochloric acid and treated with diacetyl trimer (0.5 g.). The mixture was heated for 10 min. at 90° and then the pH was readjusted to pH7 by the addition of sodium hydrogen carbonate. Examination of the reaction mixture by paper chromatography in solvent system (A) showed that there were three main products. Two of the products were the same as in the reaction with diacetyl dimer (see above) and the third was identical both in R_F value and in ultraviolet absorption data with 2,8-dihydro-4-hydroxy-6,7--dimethyl-2-oxo-8-D-ribitylpteridine ("G-compound").

The reaction mixture was next treated with enough 0.5N-sodium hydroxide to give an overall normality of 0.1N, and heated at 90° for 1 hr. The presence of riboflavin was proved by paper chromatography and ultraviolet absorption spectroscopy.
(b) 2,6-Dihydroxy-5-nitro-4-D-ribitylaminopyrimidine (0.25 g.) was dissolved in water (10 c.c.) and the nitro group reduced using sodium dithionite in alkaline solution in the usual manner. The pH of the reduced solution was adjusted to 6 by the addition of

12N-hydrochloric acid, and diacetyl trimer (0.25 g.) was added. After heating the reaction mixture at pH6 and 90° for 10 min. the pH was readjusted to pH1 by further addition of 12N-hydrochloric acid and the solution was refluxed for 30 min. On cooling riboflavin separated out as pure orange needles (0.03 g.), m.p. 289°(lit.[22], 292°).

7,8-Dihydro-2,4-dihydroxy-8-β-hydroxyethyl-6-methyl-7-oxopteridine. - 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (1 g.) in water (20 c.c.) was made alkaline by the addition of N-sodium hydroxide (10 c.c.) and was treated with sodium dithionite until the yellow solution which first went red became colourless. The mixture was then adjusted to pH5 by the addition of the required amount of 12N-hydrochloric acid, treated with pyruvic acid (100% purity; 0.8 g.), and heated at 90° for 1 hr. The reaction mixture was then filtered while still hot and left at room temperature whereupon crystals rapidly deposited. The crystals were collected and recrystallised from water to give the pteridine as long white needles (0.75 g.), m.p. >325° (Found: C,45.7; H,4.4; N,23.2. $C_9H_{10}N_4O_4$ requires C,45.4; H,4.2; N,23.5%).

7,8-Dihydro-2,4-dihydroxy-6,8-dimethyl-7-oxopteridine. - 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (1 g.) in water (30 c.c.) was made alkaline by the addition of N-sodium hydroxide (10 c.c.) and was treated with sodium dithionite until the yellow solution became colourless. The pH of the solution was next adjusted to 5 by the addition of 12N-hydrochloric acid and treated

with 100% pyruvic acid (0.75 g.). The mixture was heated for 1 hr. at 90°, filtered while still hot, and left to come to room temperature. The white crystalline product was collected, and recrystallised from water as white needles, (0.6 g.), m.p. >325°. (Found: C,46.5; H,4.2; N,27.2. $C_8H_8N_4O_3$ requires C,46.2; H,3.9; N,26.9%).

2,10-Dihydro-10-β-hydroxyethyl-2-oxo-4,6,8-trihydroxypyrimido [5,4-g]pteridine. - (a) 2,6-Dihydroxy-4-β-hydroxyethylamino-5--nitropyrimidine (1 g.) was dissolved in water (20 c.c.) and reduced using sodium dithionite in alkaline solution. When the reduction was complete the pH was adjusted to 3.5 by the addition of 12N-hydrochloric acid. The solution was next treated with alloxan (0.75 g.) and an extremely short-lived purple colouration developed. The now bright yellow solution was refluxed for 2 hr., cooled, and filtered to give a yellow solid (0.65 g.). Recrystallisation from water gave the pyrimidopteridine as bright yellow plates, m.p. >325° (Found: C,38.0; H,3.7; N,26.5. $C_{10}H_8N_6O_5.H_2O$ requires C,38.7; H,3.3; N,27.1%).

(b) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.5 g.) in hot water (12 c.c.) was treated with zinc dust (1.6 g.) and 10N-sulphuric acid (1.5 c.c.). The mixture was refluxed for 10 min. after which the zinc residues were removed by filtration of the still hot mixture. The zinc residues were washed with water (10 c.c.), and alloxan (0.4 g.) was then added to the filtrate plus washings. A deep purple colour immediately formed

and persisted during 5 min. The reaction mixture was refluxed for 2 hr. to give a bright yellow solution, which on cooling deposited the pyrimidopteridine as yellow plates (0.69 g.). Recrystallisation from water gave large lustrous yellow plates, m.p. $> 325°$.

(c) The di-formyl derivative of 5-amino-2,6-dihydroxy-4-β-hydroxyethylaminopyrimidine (0.37 g.) was dissolved in 0.1N-hydrochloric acid (50 c.c.) and the solution was refluxed for 6 hr. The volume was then reduced to 10 c.c. whereupon the pyrimidopteridine crystallised out as yellow plates (0.25 g.), m.p. $> 325°$.

(d) Barbituric acid (0.3 g.) and 2,6-dihydroxy-4-β-hydroxyethyl-amino-5-nitrosopyrimidine (0.3 g.) in glacial acetic acid (10 c.c.) and 12N hydrochloric acid (1 drop) were heated together at 120° for 9 hr. The reaction mixture was treated with ethanol and so gave a yellow precipitate which was collected and dried to give a dull yellow powder (0.25 g.). This powder contained two compounds which were separated using paper chromatography in solvent system (B). By this means the less mobile spot was shown to be identical with the products from (a), (b) and (c) in fluorescence, R_F value, and UV absorption spectrum. Repeated recrystallisation of the yellow powder from 2N hydrochloric acid gave a bright yellow solid (0.07 g.) which was identical in its infrared absorption spectrum with the products from (a), (b) and (c).

2,10-Dihydro-10-methyl-2-oxo-4,6,8-trihydroxypyrimido[5,4-g] pteridine.-(a) 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0.4 g.) was dissolved in water (25 c.c.) and reduced in the usual way using sodium hydroxide and sodium dithionite. The reduction completed the pH was adjusted to 3.5 using 12N-hydrochloric acid and alloxan monohydrate (0.25 g.) was added. The mixture became momentarily deep purple and finally bright yellow. After refluxing for 2 hr. the bright yellow solution was left to stand overnight and the resulting crystalline product (0.35 g.) was collected. Recrystallisation from water gave the pyrimidopteridine as bright yellow crystals, m.p. > 325°

(b) 2,6-Dihydroxy-4-methylamino-5-nitropyrimidine (0.35 g.) was dissolved in 98% formic acid (20 c.c.) and the hot solution was treated with zinc dust (0.5 g.). The mixture was heated at 90° for 10 min., after which the zinc salts and residues were removed by filtration of the still hot mixture. The filtrate was treated with ethanol (20 c.c.) and ether until a solid precipitated. The solid was collected and washed with ethanol and ether. The dry off-white solid was then dissolved in water (50 c.c.) containing 12N-hydrochloric acid (2 c.c.), and the solution was refluxed for 6 hr. Finally reduction of the volume of the solution to 15 c.c. gave the pyrimidopteridine as yellow crystals (0.13 g.), m.p. > 325° (Found: C, 38.2; H, 2.3; N, 29.3. $C_9H_6N_6O_4.H_2O$ requires C, 38.6; H, 2.9; N, 30.0%).

2,10-Dihydro-2-oxo-10-D-ribityl-4,6,8-trihydroxypyrimido [5,4-g]pteridine. - (a) 2,6-Dihydroxy-5-nitro-4-D-ribitylamino-pyrimidine (0.25 g.) was dissolved in water (10 c.c.) and treated with N-sodium hydroxide (5 c.c.). Sufficient sodium dithionite was next added to give a colourless solution. When the reduction was complete the pH was adjusted to 4 by the addition of 12N-hydrochloric acid and the reaction mixture was treated with alloxan (0.13 g.). The mixture was refluxed for 2 hr. and then cooled to give a bright yellow micro-crystalline product (0.16 g.). The product, recrystallised readily from water to give the pyrimidopteridine as yellow plates, m.p. >325° (Found: C,38.3; H,4.7. $C_{13}H_{14}N_6O_8 \cdot 1.5H_2O$ requires C,38.1; H,4.2%).

(b) The filtrate of the reaction of 5-amino-2,6-dihydroxy-4-D--ribitylaminopyrimidine and the dimer of 3,4-dimethyl-o-benzo-quinone (see Part III), after the removal of riboflavin, was treated with ethanol (100 c.c.). The bright yellow precipitate was twice recrystallised from water to give yellow plates (0.05 g.) identical with those obtained in (a) in all physical properties.

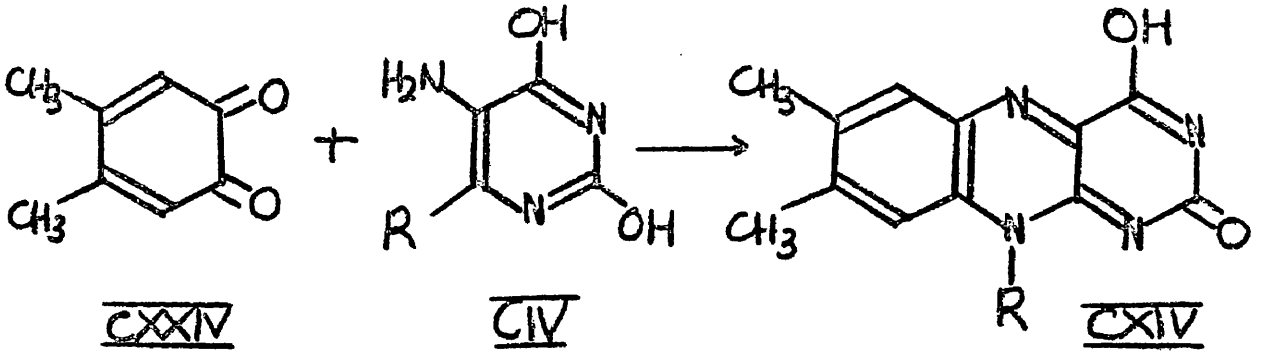
P A R T III

A New Synthesis of Riboflavin and Related Isoalloxazines.

THEORETICAL.

The availability of 5-amino-4-D-ribitylaminouraoil (CIV, R = D-ribitylamino) and analogous pyrimidine derivatives, together with the recent description[84] of a stable dimer of 3,4-dimethyl-o-benzoquinone (CXXIV) suggested a new and convenient synthesis of isoalloxazines in general.

Thus condensation of this dimer and 5-amino-2,6-dihydroxy--4-β-hydroxyethylaminopyrimidine (CIV, R = $NHCH_2CH_2OH$) gave 6,7-dimethyl-9-β-hydroxyethylisoalloxazine (CXIV, R = CH_2CH_2OH). This material was identical with a sample prepared by the literature method[114] which involves periodic acid oxidation of riboflavin (CXIV, R = D-ribityl) to the corresponding 9-formyl methylisoalloxazine (CXIV, R = CH_2CHO) which is converted using sodium borohydride to the desired 9-β-hydroxyethyl analogue (CXIV, R = CH_2CH_2OH).



CXXIV CIV CXIV

In a similar way 6,7,9-trimethylisoalloxazine (CXIV, R = CH_3, "lumiflavin") was prepared by reaction of the dimer of 3,4--dimethyl-o-benzoquinone and the 5-aminopyrimidine (CIV, R = $NHCH_3$).

Finally condensation of 5-amino-4-D-ribitylaminouracil (CIV, R = D-ribitylamino) with the dimer of 3,4-dimethyl-o--benzoquinone gave pure crystalline riboflavin.

This new method for the synthesis of isoalloxazines compares favourably with the older methods reported in the literature (see Introduction).

EXPERIMENTAL

6,7-Dimethyl-9-formylmethylisoalloxazine.[114] - Riboflavin (5 g.) suspended in 2N-sulphuric acid (130 c.c.), was treated in an ice bath with periodic acid (11 g.) in water (80 c.c.), and the mixture was stirred for 30 min., still in an ice bath. The mixture was further stirred for 2 hr. at 25° and left overnight at the same temperature to effect solution. Sodium carbonate was added to pH1.5 at which pH the solution was treated with charcoal. After the addition of a few drops of capryl alcohol the pH was further adjusted using sodium carbonate to pH3.8 and the product collected, washed with cold water, ethanol, and finally ether to give 6,7-dimethyl-9-formylmethylisoalloxazine as a bright orange powder (2.8 g.), m.p. 268-270° [lit.,[114] 270-271°].

6,7-Dimethyl-9-β-hydroxyethylisoalloxazine.[114] - (a) 6,7-Dimethyl--9-formylmethylisoalloxazine (2.8 g.) was dissolved in cold 0.1N sodium hydroxide (100 c.c.) and treated, during vigorous stirring, with sodium borohydride (0.37 g.) in water (25 c.c.). Stirring was continued for a further 2 hr. at room temperature, the solution cooled thereafter in an ice bath, treated with glacial acetic acid to pH4-4.5 and filtered. The product was washed with cold water, dilute acetic acid, and acetone to give 6,7-dimethyl-9-β-hydroxyethylisoalloxazine as an orange powder (2 g.). The isoalloxazine was purified by reprecipitation with dilute sulphuric acid from its solution in dilute sodium hydroxide and was so obtained as a bright orange powder, m.p. 300° (lit.,[114] 300-301°).

(b) 2,6-Dihydroxy-4-β-hydroxyethylamino-5-nitropyrimidine (0.25 g.) in water (10 c.c.) was made alkaline by the addition of N-sodium hydroxide (5 c.c.). Sodium dithionite was next added until the solution became colourless. The pH of the solution was reduced to 4 by the addition of the required amount of glacial acetic acid, after which a solution of the dimer of 3,4-dimethyl-o-benzoquinone (0.17 g.) in alcohol (10 c.c.) was added. The mixture was heated for 30 min. at 90° and finally made strongly acid by the addition of 12N-hydrochloric acid. On cooling pure 6,7-dimethyl-9-β-hydroxyethylisoalloxazine separated out as orange needles (0.095 g.), m.p. 297-299°.

6,7,9-Trimethylisoalloxazine ("lumiflavin"). - 2,6-Dihydroxy--4-methylamino-5-nitropyrimidine (0.1 g.) was dissolved in water (5 c.c.) and the solution was treated with 10% sodium hydroxide (5 c.c.). Sufficient sodium dithionite to decolourise the solution was next added with the solution at 90°. The pH was adjusted to 5 by the addition of a few drops of glacial acetic acid, and the mixture was treated with a solution of the dimer of 3,4-dimethyl-o-benzoquinone (0.15 g.) in alcohol (10 c.c.). Heating at 90° was continued during 30 min. after which the solution was left to stand over-night at room temperature. 6,7,9-Trimethylisoalloxazine separated out as fine orange needles (0.06g.), m.p. 325-326° (lit.[116], 328°).

6,7-Dimethyl-9-D-ribitylisoalloxazine ("Riboflavin"). - 2,6-Dihydroxy-5-nitro-4-D-Ribitylaminopyrimidine (0.25 g.) in water (10 c.c.) was made alkaline by the addition of N-sodium hydroxide (5 c.c.). Sodium dithionite was next added until the solution became colourless. The pH was reduced to 4 by the addition of the required amount of glacial acetic acid, after which a solution of the dimer of 3,4-dimethyl-o-benzoquinone (0.15 g.) in alcohol (10 c.c.) was added. The mixture was heated for 30 min. at 90° and finally made strongly acid by the addition of 12N-hydrochloric acid. On cooling pure riboflavin separated out as orange needles (0.09 g.), m.p. 288° (lit.[22] 292°), $[\alpha]_D^{25}$ - 116° in N/10 sodium hydroxide (c, 0.528) lit.,[117] $[\alpha]_D^{20}$ - 115° in N/10 sodium hydroxide (c, 0.447).

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