804. *The Hydrolysis of Ribitol* 1(5)-*Phosphate, Riboflavin* 5′-*Phosphate, and Related Compounds.*

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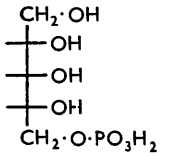
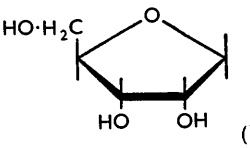
The anhydropentitol produced by acid hydrolysis of ribitol 1(5)-phosphate has been isolated and shown to be 1 : 4-anhydroribitol. The same compound is formed by acid treatment of ribitol itself. Mechanisms are proposed for these reactions.

It is suggested that so-called " lyxoflavin " from human heart muscle is a mixture of riboflavin and 2′ : 5′-anhydroriboflavin.

During work on the structure of the nucleotide, cytidine diphosphate ribitol (CDP-ribitol), it was found that ribitol 1(5)-phosphate (I) and related compounds were unexpectedly labile towards acids.[1] The products expected from mild acid hydrolysis of the nucleotide were cytidine-5′ phosphate and ribitol 1(5)-phosphate, together with smaller amounts of isomeric ribitol phosphates. The isomers would arise by acid-catalysed migration of the phosphate residue from the 1-position. It was found, however, that appreciable amounts of inorganic phosphate were liberated during hydrolysis in N-hydrochloric acid at 100°, even after short periods. Longer hydrolysis resulted in the liberation, as inorganic phosphate, of nearly all the phosphorus associated with the ribitol phosphate residue in the nucleotide. Under these conditions cytidine-5′ phosphate was largely unaffected. Synthetic D-ribitol 5-phosphate * (I) behaved similarly to CDP-ribitol, whereas the glycerophosphates and mannitol phosphate required much more vigorous conditions in order to liberate even small amounts of inorganic phosphate.



(I)



(II)

The products from ribitol 1(5)-phosphate were examined by paper chromatography. Spray reagents for phosphates and glycols revealed a complex mixture. In addition to inorganic phosphate and a spot corresponding to ribitol 1(5)-phosphate, at least three products were detected. Two of these were identified later as ribitol 2- and 3-phosphate.[2] A third product, which becomes the main component after more prolonged hydrolysis, has

* The nomenclature adopted here for polyol phosphates is discussed in ref. 2.

[1] Baddiley, Buchanan, Carss, and Mathias, *J.*, 1956, 4583.
[2] Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.

been provisionally described as an anhydroribitol. The confirmation of this structure, the mechanism of its formation, and implications in other fields are subjects of this paper.

The tentative conclusion that the main product of hydrolysis of ribitol phosphate is an anhydroribitol was based largely on its behaviour on paper chromatography. It had a higher R_F than ribitol, which suggested that it contained fewer hydroxyl groups. Also, it gave a slowly developing blue colour when the paper was sprayed with the periodate–Schiff reagents for glycols. In this respect it closely resembled a ribofuranoside and was was quite unlike an acyclic glycol, which would give a rapidly developing crimson colour.

In a larger-scale experiment the anhydroribitol was isolated in crystalline form. It contained no phosphorus, gave a tribenzoyl derivative, and readily consumed one mol. of periodate; neither formic acid nor formaldehyde was produced by the oxidation. From this and from its analysis it follows that the compound is a 1 : 4-anhydropentitol. It is interesting that other methods for the production of anhydro-compounds from polyols usually give the 5-membered rather than the 6-membered ring, the configuration of hydroxyl groups being unchanged.[3]

Although D-ribitol 5-phosphate (from D-ribose 5-phosphate) was used in these experiments, the acid-catalysed phosphate migration which occurs under the conditions employed would lead to considerable or even complete racemisation. It follows that the 1 : 4-anhydropentitol formed would be largely or entirely racemic. In a previous paper [2] a combined chemical and enzymic method was developed for the quantitative determination of D-ribitol 5-phosphate in the presence of the L-compound. It was shown by this method that racemisation of ribitol phosphates through phosphate migration was substantial in 30 minutes and complete in 90 minutes at 100° in N-hydrochloric acid. On the other hand, the formation of anhydropentitol is only complete after about 6 hours under these conditions, and it follows that only that anhydro-compound formed during the early stages of the acid treatment would have retained optical activity. This introduced difficulty in attempted comparison of the product with authentic anhydropentitols for purposes of identification.

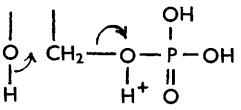
The most likely structure for the compound would be the DL-form of 1 : 4-anhydroribitol. The D-form (II) has been synthesised by the action of aqua regia on D-ribitylamine,[4] or by reduction of 2 : 3 : 5-tri-*O*-benzoyl-D-ribofuranosyl bromide with lithium aluminium hydride.[5] The product in both cases should be optically active and its relation to the product from ribitol 1(5)-phosphate would only be possible by comparison of infrared spectra. Authentic 1 : 4-anhydro-D-ribitol was prepared by the second method and converted into its 2 : 3 : 5-tri-*O*-benzoyl derivative. The infrared spectrum of this tribenzoyl compound in carbon tetrachloride solution was indistinguishable from that of the tribenzoylanhydropentitol from ribitol 1(5)-phosphate, and we consider that the two compounds are identical. The spectra of crystalline 1 : 4-anhydro-D-ribitol prepared by the second method and of our own preparation, when examined in potassium bromide discs, showed small differences in the height, but not the location, of certain peaks. These differences may arise from the fact that one preparation was a crystalline racemate whereas the other was the pure D-form.

The mechanism of formation of anhydroribitol from ribitol phosphate is probably best represented by protonation of the ester-oxygen atom at position 1(5), followed by the electronic displacements shown in (III). The hydroxyl group at position 4 in the ribitol chain is presumably in a sterically favourable situation to assist the reaction and an intramolecular nucleophilic substitution occurs. The protonated phosphoric ester is probably a common intermediate both in phosphate elimination, as above, and in phosphate migration.

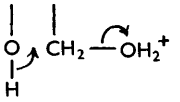
Ribitol itself was converted into 1 : 4-anhydroribitol in the presence of dilute mineral

[3] Wiggins, *Adv. Carbohydrate Chem.*, 1950, **5**, 191.
[4] Kuhn and Wendt, *Chem. Ber.*, 1948, **81**, 553.
[5] Weygand and Wirth, *ibid.*, 1952, **85**, 1000.

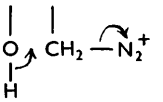
acid and, in fact, several of the experiments on the structure of the anhydro-compound were carried out with material obtained in this way. Although the reaction is much slower with the free polyol, the mechanism is probably somewhat similar. The initial stage would be the protonation of the primary hydroxyl group and this would be followed by the electronic displacements shown in (IV). It might be expected that the phosphate would yield the anhydro-compound more rapidly than does the free polyol, since the electronegative properties of the phosphorus atom would assist the reaction.



(III)



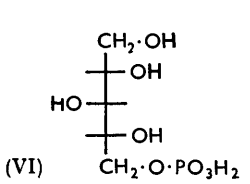
(IV)



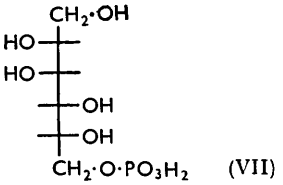
(V)

The mechanism of formation of anhydroribitol from ribitol or its phosphate is similar to the mechanism of its formation from ribitylamine in the presence of nitrosyl chloride (aqua regia). It is well known that sterically suitable amino-alcohols are converted into anhydro-compounds rather than glycols by the action of nitrous acid.[4,6] We consider that this occurs by electronic displacements in the intermediate diazonium compound as shown in (V). In the reaction with ribitylamine, the suitably placed hydroxyl group at position 4 is involved in an intramolecular nucleophilic displacement very similar to the one which occurs with the conjugate acids from ribitol or its phosphate.

The hydrolysis of ribitol 1(5)-phosphate at pH 4 proceeded readily and normally, giving ribitol and inorganic phosphate. The mechanism of hydrolysis of phosphoric esters at pH 4 differs from that operating in stronger acid.[7] At the higher pH the reacting species is the monoanion and hydrolysis will proceed with phosphorus–oxygen fission.[8] No migration of the phosphate group was observed under these conditions. The hydrolysis of glycerophosphates also occurs readily at pH 4, and it has been shown that no phosphate migration takes place during their hydrolysis.[9]



(VI)



(VII)

It has not yet been possible to examine an extensive range of polyol phosphates in respect of their acid hydrolysis. However, D-xylitol 5-phosphate (VI) was readily converted into inorganic phosphate and an anhydro-compound. Qualitative data from paper chromatograms indicated that this polyol phosphate was converted into an anhydro-compound at a rate only slightly slower than that of ribitol 1(5)-phosphate. In these experiments it was necessary to determine the extent of reaction from the amount of unchanged polyol phosphate present. The periodate–Schiff method is nearly equally sensitive towards all the polyols and their phosphates, but is much less sensitive towards anhydro-compounds containing a *trans*-glycol system. D-Mannitol 1(6)-phosphate (VII)

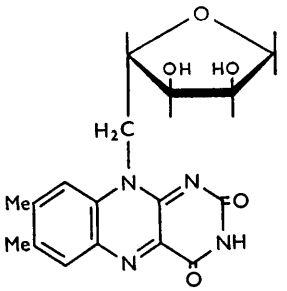
[6] Wiggins, *Nature*, 1946, **157**, 300; Bashford and Wiggins, *J.*, 1948, 299; Foster, *Chem. and Ind.*, 1955, 627.
[7] Desjobert, *Compt. rend.*, 1947, **224**, 575; *Bull. Soc. chim. France*, 1947, **14**, 809.
[8] Butcher and Westheimer, *J. Amer. Chem. Soc.*, 1955, **77**, 2420.
[9] Bailly, *Bull. Soc. chim. France*, 1942, **9**, 340.

was more stable towards hydrolysis than the two pentitol phosphates. It appears, however, that in the three cases so far examined the phosphates yield anhydro-compounds more readily than do the free polyols, and the latter are not intermediates in the reaction.

In the following paper [10] it is shown that erythritol and all the pentitols and hexitols are converted to varying extents into anhydro-compounds, or mixtures of anhydro-compounds, when they are heated in dilute acid, and this has been made the basis of a useful paper-chromatographic procedure for the identification of polyols. It was found that reaction occurred most readily with those polyols in which at least three consecutive secondary hydroxyl groups are conventionally depicted on the same side of the polyol chain. The differences in the reaction rates are probably less clearly observable in the polyol phosphates. Even here, however, a considerable difference in rate is observed with the phosphates of ribitol and mannitol.

In view of the ease of formation of 1 : 4-anhydroribitol from ribitol 1(5)-phosphate, it seems likely that phosphoric esters of other polyhydroxy-compounds might behave similarly during hydrolysis. Consequently, we have re-examined the acid hydrolysis of riboflavin 5′-phosphate. It has been assumed hitherto that acid hydrolysis of this well-known natural product proceeds normally, to give riboflavin and inorganic phosphate. Synthetic riboflavin 5′-phosphate (kindly supplied by Professor F. Bergel) has now been hydrolysed at pH 4 and in N-hydrochloric acid, and the products have been examined by paper chromatography. At pH 4 hydrolysis of the monoanion was normal, the only products being riboflavin and inorganic phosphate. At the lower pH hydrolysis gave inorganic phosphate together with a mixture of riboflavin and a greater amount of a product with a higher R_F than riboflavin in basic solvents. The new product was not isolated in a pure state but it is probably a 2′ : 5′-anhydro-compound with the structure (VIII). Under similar conditions, riboflavin itself gave a very small amount of the anhydro-compound, together with traces of unidentified products with low R_F values. If the ease of formation of anhydro-compounds from polyol phosphates is largely governed

(VIII)



by steric factors in the polyol chain, then it is likely that the bulky flavin residue in riboflavin 5′-phosphate would interfere with the displacement of phosphate by the 2′-hydroxyl group. Consequently, anhydro-compound formation would be retarded and some normal hydrolysis to riboflavin would occur.

An anhydroriboflavin has been reported previously.[11] This was obtained by the spontaneous decomposition of 5′-toluene-*p*-sulphonylriboflavin and it was assumed to be a 4′ : 5′-anhydro-compound. It was not possible at the time to eliminate alternative structures, and it is not known whether this compound differs from the anhydroriboflavin obtained from the phosphate.

It is possible that during certain biochemical and nutritional studies on vitamin B_2

[10] Baddiley, Buchanan, and Carss, following paper.
[11] Forrest, Mason, and Todd, *J.*, 1952, 2530.

(riboflavin) acidic conditions may have resulted in the partial conversion of either riboflavin 5′-phosphate (FMN) or of flavin–adenine dinucleotide (FAD) into 2′ : 5′-anhydroriboflavin. This may well have led to occasional confusion in the interpretation of results. For example, prolonged conditions of acidity were employed during the isolation of so-called " lyxoflavin " from human heart muscle.[12] The reliability of the methods used for the identification of this compound has been questioned previously [13] and its homogeneity has not been established by modern techniques. We consider it likely that this material is a mixture of riboflavin and 2′ : 5′-anhydroriboflavin, which would have arisen by acidic hydrolysis of riboflavin 5′-phosphate and its nucleotide present in the fresh heart muscle.

Experimental

1 : 4-*Anhydroribitol from* D-*Ribitol* 5-*Phosphate.*—A solution of the barium salt of D-ribitol 5-phosphate (60 mg.) in water (1 ml.) was passed through a column of Dowex-50 resin (H^+ form). Dilute hydrochloric acid was added to the acidic eluate to give an approximately N-solution (total volume *ca.* 3 ml.). The solution was heated in a sealed tube at 100° for 20 hr. Samples were removed at intervals and examined by paper chromatography in solvent system *A*. Unchanged material and anhydro-compound were detected by the periodate–Schiff reagents for glycols.[14] Solvent was removed *in vacuo* and the oily residue, which consisted largely of anhydro-compound and inorganic phosphate, was benzoylated directly with benzoyl chloride in pyridine. The crystalline tribenzoyl derivative, m. p. 113—114°, was isolated in the usual manner and was recrystallised from ethanol. It was indistinguishable from tri-*O*-benzoyl-anhydroribitol, m. p. and mixed m. p. 113—114°, prepared from ribitol by the method described below.

1 : 4-*Anhydroribitol from Ribitol.*—A sealed tube containing ribitol (1·5 g.) in 2N-hydrochloric acid (100 ml.) was heated in a water-bath at 100° for 66 hr. Solvent was evaporated and residual hydrochloric acid was removed by repeated evaporation with water. *Anhydroribitol* was recrystallised from benzene containing a little alcohol, by adding dry ether. The hygroscopic needles, m. p. 74—75°, were dried *in vacuo* at 65° (Found: C, 44·4; H, 7·5. $C_5H_{10}O_4$ requires C, 44·8; H, 7·5%). The product was indistinguishable from authentic 1 : 4-anhydroribitol when examined by paper chromatography in solvent systems, *A*, *B*, and *C* (see below).

Periodate Oxidation.—This was carried out in the usual manner with sodium metaperiodate. Oxidation was complete in 10 min. at room temperature. One equivalent of anhydroribitol consumed 1·02 equivalents of periodate and no further uptake was observed after 24 hr. No formic acid was formed (titration with sodium hydroxide in the presence of methyl-red). Less than 0·05 mol./mol. of formaldehyde was detected in the oxidation mixture by Nash's method.[15]

2 : 3 : 5-*Tri*-O-*benzoylanhydroribitol.*—The crystalline anhydroribitol was benzoylated with benzoyl chloride in pyridine. Recrystallised from alcohol, *tri*-O-*benzoylanhydroribitol* had m. p. 114° (Found: C, 70·0; H, 5·0. $C_{26}H_{21}O_7$ requires C, 70·1; H, 4·7%). The tri-*O*-benzoyl derivative of anhydroribitol prepared from D-ribose by Weygand and Wirth's method [5] did not crystallise but was purified by chromatography on neutral alumina, elution being carried out with benzene.

Hydrolysis of D-*Ribitol* 5-*Phosphate at* pH 4.—Samples (*ca.* 1 mg.) of the barium salt of D-ribitol 5-phosphate were treated with a little Dowex-50 (H^+ form) resin in water and ammonium acetate was added to pH 4 (approx. 0·2M). The resulting solutions were heated in sealed tubes at 100°, and samples were withdrawn after 20 and 46 hr. The products were examined by paper chromatography in solvent system *A*. Hydrolysis was complete after 20 hr., the products being inorganic phosphate and ribitol. Anhydroribitol and ribitol 2(4)- and 3-phosphate were not formed.

[12] Pallares and Gaza, *Arch. Biochem.*, 1949, **22**, 63.
[13] Emerson and Folkers, *J. Amer. Chem. Soc.*, 1951, **73**, 5383.
[14] Buchanan, Dekker, and Long, *J.*, 1950, 3162; cf. ref. 10.
[15] Nash, *Biochem. J.*, 1953, **55**, 416.

D-*Xylitol 5-Phosphate* (Preparation by Mr. C. P. FAWCETT).—D-Xylose 5-phosphate [16] was reduced in aqueous solution with sodium borohydride and the *barium salt* of xylitol phosphate was isolated by methods described before [2] for ribitol phosphate (Found: P, 8·3. $C_5H_{11}O_8PBa$ requires P, 8·4%). The product was homogeneous when examined by paper chromatography.

D-*Mannitol* 1(6)-*Phosphate*.—A sample isolated from natural sources [17] and a synthetic sample, kindly supplied by Dr. N. O. Kaplan, were used.

Hydrolysis of Xylitol Phosphate and Mannitol Phosphate.—The barium salts were converted into free acids by passage of their solutions through Dowex-50 (H^+ form) resin columns, and hydrolysis was carried out in 2N-hydrochloric acid as for ribitol phosphate. Xylitol phosphate was decomposed slightly less rapidly than ribitol phosphate, whereas mannitol phosphate was only affected to a small extent. The phosphorus-free products were identical with those obtained from the respective polyols after acid treatment.[10]

Hydrolysis of Riboflavin 5'-Phosphate.—Samples of riboflavin and its phosphate in N-hydrochloric acid were heated in sealed tubes at 100° in the dark for 24 hr. After evaporation of solvent the samples were examined by paper chromatography in solvent system *A* in the dark. Products were detected by spray reagents for phosphates and by inspection in ultraviolet light.

Paper Chromatography.—Ascending-front chromatography (see Table) was carried out on Whatman No. 4 paper which had been washed with 2N-acetic acid, then water. The following solvents systems were used: *A*, *n*-propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1); *B*, pyridine–ethyl acetate–water (2 : 7 : 1); *C*, *iso*butyric acid–0·5N-ammonia (10 : 6 ml.).

	R_F in solvents.			
	A	*B*	*C*	IO_4^-–Schiff reaction
D-Ribitol 5-phosphate	0·30	—	—	Rapid, magenta
D-Xylitol 5-phosphate	0·30	—	—	,,
D-Mannitol 1(6)-phosphate	0·29	—	—	,,
Ribitol	0·62	—	—	,,
Xylitol	0·61	—	—	,,
D-Mannitol	0·59	—	—	,,
1 : 4-Anhydroribitol	0·71	0·90	0·64	Slow, blue
Anhydroxylitol	0·72	—	—	,, faint
Anhydromannitol	0·68	—	—	Rapid, yellow
Riboflavin	0·41	—	—	No reaction
Riboflavin 5'-phosphate	0·20	—	—	,,
Anhydroriboflavin	0·50	—	—	,,

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[16] Barnwell, Saunders, and Watson, *Canad. J. Chem.*, 1955, **33**, 711; Gorin, Hough, and Jones, *J.*, 1955, 585.
[17] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **64**, 599.