642. *Nucleotides. Part V. Riboflavin-5′ Phosphate.*

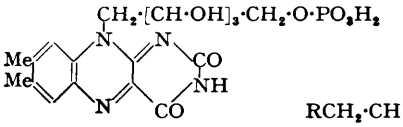
By H. S. Forrest and A. R. Todd.

Treatment of riboflavin with phosphoryl chloride in pyridine containing a small amount of water yields a cyclic phosphate, riboflavin-4′ : 5′ phosphate. Acid hydrolysis of this cyclic ester yields riboflavin-5′ phosphate, identical with the naturally occurring riboflavin phosphate, and provides a preparative method for this co-enzyme.

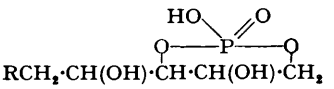
The yellow co-enzyme which takes part in the oxidation of glucose-6 phosphate in biological systems (Warburg and Christian, *Naturwiss.*, 1932, **20**, 688; *Biochem. Z.*, 1932, **254**, 438) was first isolated in a pure state by Theorell (*Biochem. Z.*, 1934, **272**, 155; 1935, **275**, 344), who showed that it contained one atom of phosphorus and moved towards the anode in cataphoresis experiments. On the basis of his studies he concluded that the co-enzyme was probably a riboflavin phosphate. Shortly thereafter, Kuhn and Rudy (*Ber.*, 1935, **68**, 383) reported a synthesis of the co-enzyme by direct phosphorylation of riboflavin with phosphoryl chloride. The claim that the natural product was riboflavin-5′ phosphate (I) rested on a further synthesis by the route: 5′-trityl riboflavin ⟶ 2′ : 3′ : 4′-triacetyl 5′-trityl riboflavin ⟶ 2′ : 3′ : 4′-triacetyl riboflavin ⟶ riboflavin-5′ phosphate (Kuhn, Rudy, and Weygand, *Ber.*, 1936, **69**, 1543). Support for this formulation was also provided by Karrer, Frei, and Meerwein (*Helv. Chim. Acta*, 1937, **20**, 79), who showed that treatment of the natural co-enzyme with periodate gave no formaldehyde, indicating that the 4′ or 5′ position was blocked. Since then, little chemical work has been done on the co-enzyme and it has remained a rather inaccessible substance.

We have re-investigated the direct phosphorylation of riboflavin reported by Kuhn and Rudy

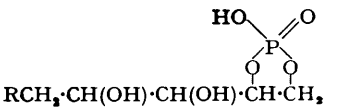
(*loc. cit.*) and have found that the reaction is more complex than was at first thought, although it can be made the basis of a relatively convenient preparative method for riboflavin-5′ phosphate. Treatment of riboflavin in anhydrous pyridine with phosphoryl chloride at room temperature yielded a brownish flocculent material of high molecular weight from which, by vigorous acid hydrolysis, a small amount of a product could be obtained whose behaviour on paper chromatography was identical with that of the natural riboflavin phosphate. If, on the other hand, phosphoryl chloride (2 mols.) was added to a solution of riboflavin (1 mol.) in pyridine containing a small amount of water (1—4 mols.) there was immediate deposition of a flocculent yellow precipitate which contained phosphorus but was not identical with natural riboflavin phosphate. The same product was obtained by an exact repetition of the experimental procedure given by Kuhn and Rudy (*loc. cit.*). Considerable difficulty was encountered in the purification of this product which was not homogeneous. In the crude state, or after partial purification *via* the brucine salt, it was unaffected by sodium metaperiodate and this fact, coupled with the apparent identity (paper chromatogram) of its chief component with a riboflavin derivative obtained by treating natural flavin-adenine dinucleotide with ammonia, led us at first to believe that it might be essentially a cyclic phosphate of riboflavin in which the phosphate residue was attached to the 3′ and 5′ positions as in (II) :



(I.)



(II.)



(III.)

Further purification of the synthetic material by the chromatopile technique (Mitchell and Haskins, *Science*, 1949, **110**, 278), however, yielded the pure cyclic ester which on oxidation with periodate consumed 1 mol. of oxidant per mol. of ester without production of formaldehyde and which must, therefore, be formulated as riboflavin-4′ : 5′ phosphate (III). The curious stability of the crude phosphorylation product to periodate may possibly be due to the presence of excess of phosphate (cf. Bell, *J.*, 1948, 992) which is removed in the purification process. Cyclic esters of phosphoric acid have, of course, been previously made from glycols and their existence as intermediates has been postulated in various rearrangements. Thus, Farrar (*J.*, 1949, 3131) has postulated an intermediate of this type to explain the ready hydrolysis of glucose-2 phosphate. A similar hypothesis has been advanced to explain the conversion of glycerol-2 phosphate into glycerol-1 phosphate (Verkade *et al.*, *Rec. Trav. chim.*, 1940, **59**, 886), a reaction which Chargaff (*J. Biol. Chem.*, 1942, **144**, 455) has shown to be intramolecular by radioactive tracer studies.

Acid hydrolysis of the crude cyclic phosphate gave, as the main product, a monophosphate of riboflavin which on paper chromatograms appeared to be identical with natural riboflavin phosphate. There were, however, at least two other substances present in the crude hydrolysed material, which, although it gave analytical values close to those required for riboflavin phosphate, consumed only 1·6 mols. of periodate per mol. of ester, even after repeated recrystallisation from water. One of the impurities was identified, not unexpectedly, as riboflavin; the second could not be separated in sufficient quantity to permit of structural determination, but from its general behaviour it seemed to be a polyphosphate. Pure riboflavin-5′ phosphate was prepared from the crude hydrolysed product by using a chromatopile; on periodate titration it consumed 2 mols. of oxidant per mol. and gave no formaldehyde. For preparative purposes there appears to be no advantage in purifying the cyclic ester (II) before acid hydrolysis, since the major by-product in either case is riboflavin. It should be noted that the use of periodate oxidation in the structural elucidation of riboflavin derivatives is complicated by the fact that riboflavin itself shows an excessive uptake of periodate at room temperature : the reason for this anomalous behaviour is at present being investigated. This complication can, however, be avoided by carrying out all periodate oxidations at 0°, at which temperature riboflavin and its acyl derivatives behave normally.

In connection with other work directed towards a synthesis of flavin-adenine dinucleotide, we have repeated the work of Kuhn *et al.* (*loc. cit.*) on the preparation of riboflavin-5′ phosphate from 2′ : 3′ : 4′-triacetyl riboflavin. The latter substance was found to show anomalous melting-point behaviour, different samples showing variable m. p.s between 180° and 194°, although they appeared quite homogeneous on chromatography. The 5′-hydroxy-group was also found to be curiously inert, being unaffected by trityl chloride, toluene-*p*-sulphonyl

chloride, or dibenzyl chlorophosphonate under the usual reaction conditions; it did, however, react with phosphoryl chloride, and treatment of the triacetyl compound with this reagent in pyridine solution gave a product which after deacetylation gave a poor yield of riboflavin-5′ phosphate.

The work described in this paper has depended to a large extent on the application of paper chromatographic techniques. Originally we used the butanol–acetic acid–water solvent system established by Crammer (*Nature*, 1948, **161**, 349) for compounds of the type described, but it suffers from the defect that in it the phosphorus compounds have low R_F values. A large number of alternative systems were investigated based on the work of Hanes and Isherwood (*Nature*, 1949, **164**, 1107). Of these, the system *tert.*-butanol–pyridine–water was selected as the most useful, and used in conjunction with the aqueous disodium hydrogen phosphate–*iso*amyl alcohol system described by Carter (*J. Amer. Chem. Soc.*, 1950, **72**, 1466) it permits ready identification of the phosphorylated flavin derivatives described in this paper. Other solvent systems are described in the experimental section where they were applied. No R_F values are quoted because the *tert.*-butanol–pyridine–water system is sensitive to temperature changes over which no control was exercised, the appropriate reference compounds being always run simultaneously on the same paper.

The experiments here described give final confirmation of the view that the natural " flavin mononucleotide " is riboflavin-5′ phosphate and provide a method for its small-scale preparation. A method very similar in type, but more convenient on a larger scale, has been developed independently in the laboratories of Hoffmann–La Roche Inc., Nutley, New Jersey. We should like to express our thanks to Drs. L. A. Flexser and W. G. Farkas for their kindness in exchanging information on this matter with us.

The finding of (II) as a product of the degradation of natural flavin-adenine dinucleotide with ammonia is of some interest. If the accepted structure of this co-enzyme is correct (Warburg and Christian, *Biochem. Z.*, 1938, **298**, 150) it must presumably arise through an intramolecular phosphorylation. The observation may be of some value not only in the chemistry of flavin-adenine dinucleotide itself, but also in that of Sanadi and Huenneken's new flavin dinucleotide (flavin *X*) (Abstr. Amer. Chem. Soc. Meeting, April 1950, p. 60C).

Experimental.

Reaction of Riboflavin with Phosphoryl Chloride.—(a) *In dry pyridine.* Riboflavin (260 mg.) was dissolved by boiling in carefully dried pyridine (130 c.c.), and to the cooled (room temperature) solution was added phosphoryl chloride (0·2 c.c.) in dry pyridine (10 c.c.). The solution gradually darkened and deposited a dark-brown precipitate. This was collected by centrifugation, washed with ethanol and acetone, and dried to give a brownish-yellow hygroscopic solid (300 mg.). This material (200 mg.) was heated with hydrochloric acid (7 c.c.; 3N.) for one hour on the water-bath. The solution was then cooled and neutralised with sodium hydroxide, and acetic acid added to pH 4. Charcoal (500 mg.) was then added to adsorb all the riboflavin derivatives, and after being shaken for 1 hour the charcoal was collected, washed thoroughly with water, and eluted by being shaken with pyridine–methanol–water (in equal proportions). The yellow eluate so obtained was evaporated to dryness, and the residue dissolved in a little water and then treated with ethanol and acetone. The flocculent yellow precipitate (20 mg.) was collected by centrifugation and dried. On a paper chromatogram (*tert.*-butanol–pyridine–water), the product gave a spot corresponding to natural riboflavin phosphate, together with an unidentified, faster-moving spot.

(b) *In pyridine containing water.* Numerous experiments were carried out to determine the best conditions for reaction, involving addition of various proportions of water before and after addition of the phosphoryl chloride–pyridine. Finally, the following procedure was adopted: To riboflavin (190 mg.) in dry pyridine (100 c.c.) was added phosphoryl chloride (0·09 c.c.) in pyridine (5 c.c.) followed immediately by water (0·009 c.c.). The flocculent precipitate (170 mg.) which formed was collected after one hour by centrifugation, and after being dried *in vacuo* it was converted into the sodium or calcium salt by dissolution in water, addition of sodium or calcium acetate, and precipitation of the salts with ethanol. Neither of these products, on treatment with sodium metaperiodate (0·238M.) in aqueous solution, consumed any of the oxidising agent over a period of 6 days. A benzidine salt, prepared either from the sodium salt after its conversion into the free acid by means of a cation-exchange column, or directly from the above precipitate by addition of an ethanolic benzidine solution to a concentrated aqueous solution of the flavin, gave anomalous periodate uptakes of 7—10 mols./mol., due presumably to oxidation of the benzidine moiety. Attempts to purify the *benzidine* salt by crystallisation resulted in considerable loss, but a once-recrystallised sample gave a satisfactory analysis for a hydrated salt of a riboflavin monophosphate (Found: C, 49·1; H, 5·5; N, 10·3. $C_{17}H_{21}O_9N_4P,C_{12}H_{12}N_2,4H_2O$ requires C, 48·9; H, 5·8; N, 11·8%). The regenerated free acid, however, gave very indifferent analytical values (Found: C, 35·3; H, 4·6; N, 10·6%).

Before these analytical values were obtained, the free acid was chromatographed on paper by using Crammer's *n*-butanol–acetic acid–water system (*loc. cit.*); the main component was a substance which travelled *in this system only* at the same speed as natural riboflavin phosphate, but it also contained some

riboflavin and left a considerable residue at the origin of the spot. This residue was at the time thought to be due to adsorption of the acid on the paper (cf. Hais and Pecakova, *Nature*, 1949, **163**, 768) but the poor analytical values later obtained for the acid indicate that it was probably due to some impurity.

Riboflavin-4′ : 5′ Phosphate.—The dried flocculent precipitate (170 mg.), obtained in Experiment (*b*) above, was dissolved in water, and the solution was adsorbed on ten Whatman No. 1 filter circles (9 cm.). These were then dried and placed in a chromatopile using 800 of the same circles and developing the pile with *n*-propanol–pyridine–water (1 : 3 : 1; 500 c.c.). After 36 hours, two bands had appeared as well as a residue at the origin. These two bands were washed off the paper with a large excess of water at room temperature and tested on an ordinary paper chromatogram. The faster-moving band was thus shown to be riboflavin and the slower the desired product. Evaporation *in vacuo* of the aqueous solution of this latter to a small bulk, followed by treatment of the concentrated solution with *n*-propanol and acetone, caused precipitation of a flocculent yellow solid which was collected by centrifugation (60 mg.). This, on treatment with sodium metaperiodate (0·236M.) at 0°, consumed 0·92 mol./mol. in 8 hours, after which no further oxidation took place. The analysis, however, was still unsatisfactory and the chromatopile product (60 mg.) was therefore redissolved in water, the solution passed through a cation-exchange column (Zeo-Karb 215), and the microcrystalline *phosphate* (40 mg.) re-isolated in the usual way (Found: C, 46·1; H, 5·0; N, 12·5. $C_{17}H_{19}O_8N_4P$ requires C, 46·4; H, 4·3; N, 12·8%).

Action of Ammonia on Natural Flavin-adenine Dinucleotide.—While surveying possible solvent systems for riboflavin phosphates, it was noticed that in solvents containing ammonia, natural flavin-adenine dinucleotide (obtained as a dilute aqueous solution through the courtesy of Mr. L. G. Whitby), gave two spots. Accordingly, a dilute solution of the co-enzyme was treated with an equal volume of concentrated ammonia (*d* 0·88) and after 24 hours at room temperature, the solution was used for further investigation in solvent systems free from ammonia. In this way it was shown that the natural co-enzyme was partially split to a substance moving at the same speed as the cyclic phosphate obtained as above in the following solvent systems: *tert.*-butanol–pyridine–water (60 : 15 : 25), *n*-propanol–pyridine–water (20 : 60 : 20), *n*-propanol–ammonia–water [60 : 30 (*d* 0·88) : 10], and *tert.*-butanol–pyridine–water (50 : 15 : 35).

Riboflavin-5′ Phosphate by Acid Hydrolysis of Riboflavin-4′ : 5′ Phosphate.—The dried crude phosphorylation product (200 mg.) was treated with hydrochloric acid (0·1N.; 20 c.c.) for one hour on the water bath. The solution was then concentrated *in vacuo* to a small bulk, and ethanol and acetone were added to precipitate a yellow solid which was collected and dried (140 mg.). Paper chromatography of this in the previously mentioned solvent systems, as well as in *n*-propanol–*N*-methylmorpholine–water (60 : 15 : 25), *n*-propanol (or *tert.*-butanol)–1%-aqueous boric acid (80 : 20), and Carter's disodium hydrogen phosphate system (*loc. cit.*) gave as the main spot a substance moving at the same speed as natural riboflavin phosphate. The analysis was satisfactory (Found: C, 44·8; H, 4·7; N, 12·4. Calc. for $C_{17}H_{21}O_9N_4P$: C, 44·7; H, 4·6; N, 12·4%), but on treatment with sodium metaperiodate in aqueous solution at 0°, it consumed only 1·6 mols. of oxidant/mol. It could be crystallised, with considerable loss, from water or, better, N-hydrochloric acid, but this process did not affect the periodate uptake.

The crude product (150 mg.) was therefore dissolved in water and readsorbed on filter papers as before. The chromatopile was developed with propanol–pyridine–water (20 : 60 : 20) and after 36 hours the main band was eluted by washing with water. The pure material was obtained by evaporation to a small bulk *in vacuo* and precipitation with ethanol and acetone. It was finally recrystallised from N-hydrochloric acid, whereupon it was obtained as a microcrystalline yellow solid (Found: C, 45·0; H, 5·0; N, 12·4%). On treatment at 0° with sodium metaperiodate it consumed 1·95 mols. of oxidant/mol., and was identical in chromatographic behaviour with natural riboflavin phosphate.

Repetition of Kuhn and Rudy's Phosphorylation Procedure (loc. cit.).—Riboflavin (100 mg.) in dry pyridine (50 c.c.) was treated at 0° with phosphoryl chloride (0·025 c.c.) in pyridine (3 c.c.). After one hour at this temperature and eight hours at room temperature, a flocculent yellow precipitate had appeared. This was collected in the usual way and dried (30 mg.); it was shown by paper chromatography to be identical with the crude cyclic riboflavin phosphate obtained above. The pyridine mother-liquors were evaporated to dryness and the residue, in a small amount of water, treated with ethanol and acetone to give a yellow solid (30 mg.), shown by paper chromatography to consist of riboflavin, the above cyclic phosphate, and a third, unidentified, constituent.

2′ : 3′ : 4′-Triacetyl Riboflavin.—This was prepared through the 5′-trityl riboflavin, as previously mentioned, followed by acetylation and removal of the trityl group. Purification of the 5′-trityl compound was achieved by preliminary treatment of the crude product with *cyclo*hexene (to remove triphenylcarbinol), then exhaustive extraction with ethyl acetate, and finally recrystallisation of the product from absolute ethanol. The material thus obtained softened at 170°, darkened at 240°, and finally melted at 246—248°; but it decomposed immediately when placed in an apparatus heated to 220°.

The triacetyl riboflavin ran as a single spot on paper chromatograms in a large number of solvent systems and was not altered or resolved by chromatography on alumina (neutral) with ethyl acetate as solvent, followed by elution with increasing concentrations of methanol; yet products from different preparations had m. p.s between 178—180° and 192—194° (Found: C, 54·7; H, 5·3; N, 11·4. Calc. for $C_{23}H_{26}O_9N_4$: C, 55·0; H, 5·2; N, 11·1%).

Attempted Reaction of 2′ : 3′ : 4′-Triacetyl Riboflavin with Trityl Chloride.—An experiment, typical of many with toluene-*p*-sulphonyl chloride, dibenzyl chlorophosphonate, and trityl chloride, was as follows: The triacetyl compound (50 mg.; m. p. 183—185°) was heated in dry pyridine (10 c.c.) with trityl chloride (170 mg.) for two hours on the water-bath. After cooling, the reaction mixture was poured into water, and the fluorescent material extracted with ethyl acetate. The extract was washed in turn with

dilute hydrochloric acid, sodium hydrogen carbonate solution, and water, and then dried. It was then passed through a column of neutral alumina, and a single band was formed and eluted with methanol. Evaporation of the eluate and recrystallisation of the residue from alcohol gave a yellow solid, m. p. and mixed m. p., with the starting material, 180—182°.

Reaction of Phosphoryl Chloride with 2′ : 3′ : 4′-Triacetyl Riboflavin.—The triacetyl compound (100 mg.; m. p. 190—192°) in pyridine (15 c.c.) was treated with a large excess of phosphoryl chloride (1 c.c.) in pyridine (15 c.c.). The solution rapidly darkened. After two minutes it was poured on ice, and the whole then evaporated to dryness. The residue was dissolved in water and adsorbed on charcoal (0·5 g.), which was collected and thoroughly washed with water. Riboflavin derivatives were then eluted with a pyridine–methanol–water mixture (100 c.c.) and the eluate was evaporated to dryness. The residue was allowed to stand with aqueous ammonia [5 c.c. (*d* 0·88) +5 c.c. water] for 24 hours. The resulting solution was shown to contain as its main constituent a product running at the same speed as natural riboflavin phosphate on paper chromatograms. [A crude brucine salt (70 mg.) was obtained from the main bulk by treatment of the evaporated residue with brucine (100 mg.) in chloroform.]

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